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(54) **MICROORGANISMS FOR THE PRODUCTION OF 1,4-BUTANEDIOL AND RELATED METHODS**

(75) Inventors: **Stephen J. Van Dien**, San Diego, CA (US); **Anthony P. Burgard**, Bellefonte, PA (US); **Robert Haselbeck**, San Diego, CA (US); **Catherine J. Pujol-Baxley**, San Diego, CA (US); **Wei Niu**, Lincoln, NE (US); **John D. Trawick**, San Diego, CA (US); **Harry Yim**, San Diego, CA (US); **Mark J. Burk**, San Diego, CA (US); **Robin E. Osterhout**, San Diego, CA (US); **Jun Sun**, San Diego, CA (US)

(73) Assignee: **Genomatica, Inc.**, San Diego, CA (US)

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Primary Examiner — Paul Holland
(74) *Attorney, Agent, or Firm* — Jones Day

(57) **ABSTRACT**

The invention provides non-naturally occurring microbial organisms comprising a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO and further optimized for expression of BDO. The invention additionally provides methods of using such microbial organisms to produce BDO.

23 Claims, 61 Drawing Sheets

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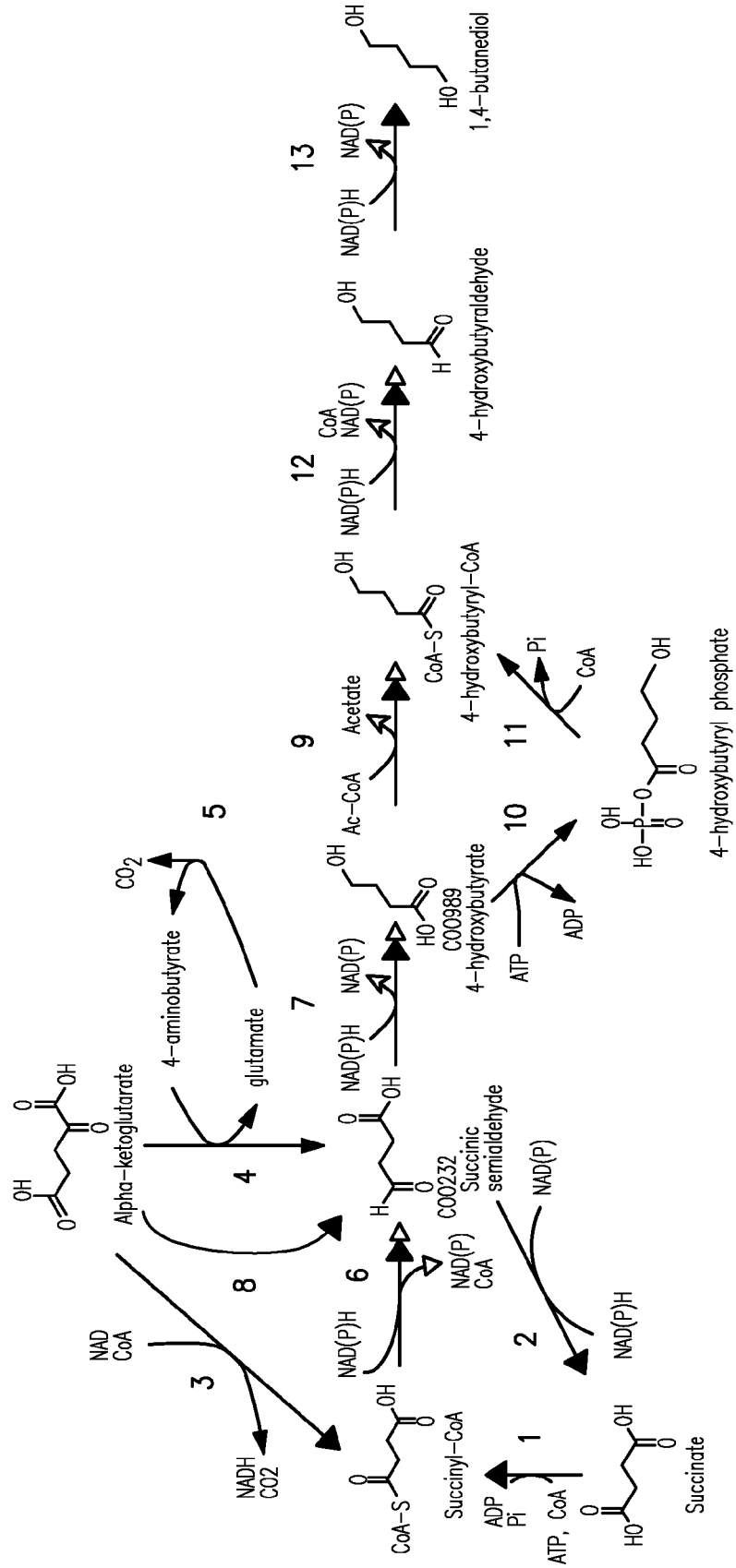

Succinate
Succinyl-CoA
Succinic semialdehyde
Alpha-ketoglutarate
4-aminobutyrate
glutamate
4-hydroxybutyrate
4-hydroxybutyryl-CoA
4-hydroxybutyraldehyde
1,4-butanediol
4-hydroxybutyryl phosphate
Acetate
Ac-CoA
ATP
ADP
Pi
CoA
NAD
NADH
CO2
NAD(P)
NAD(P)H
CO0232
CO0989
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 1

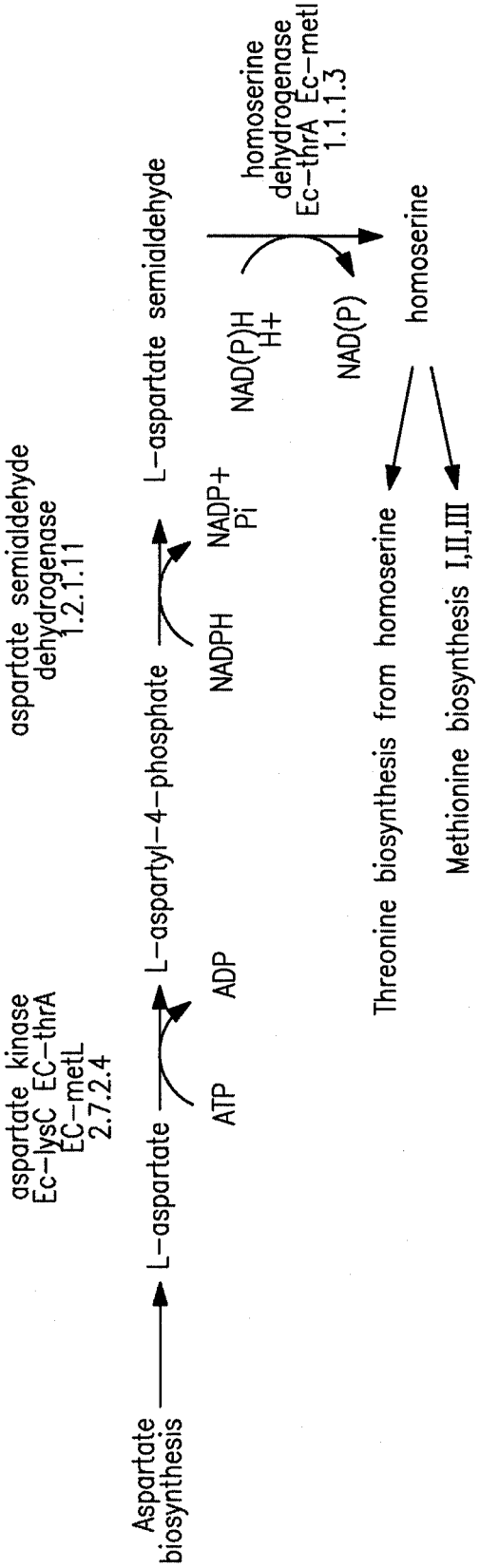

Aspartate biosynthesis
L-aspartate
aspartate kinase Ec-lysC EC-thrA EC-metL 2.7.2.4
ATP
ADP
L-aspartyl-4-phosphate
aspartate semialdehyde dehydrogenase 1.2.1.11
NADPH
NADP+ Pi
L-aspartate semialdehyde
homoserine dehydrogenase Ec-thrA Ec-metl 1.1.1.3
NAD(P)H H+
NAD(P)
homoserine
Threonine biosynthesis from homoserine
Methionine biosynthesis I,II,III

FIGURE 2

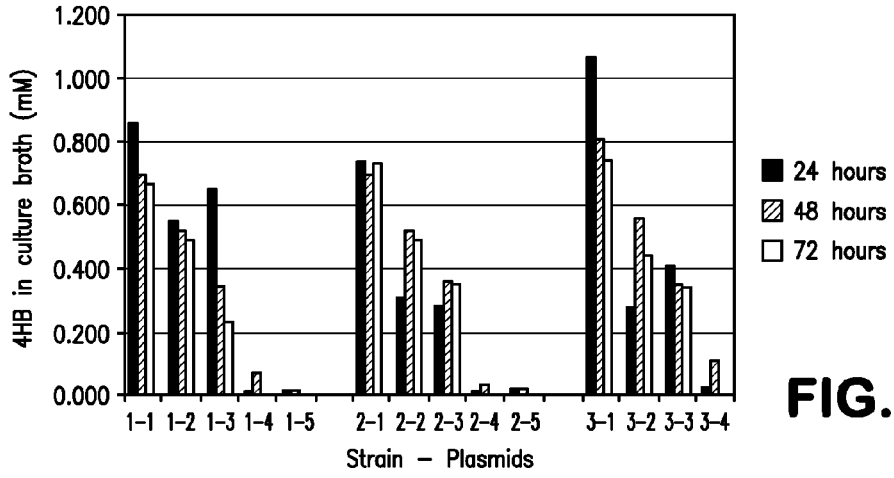


FIG. 3(a)

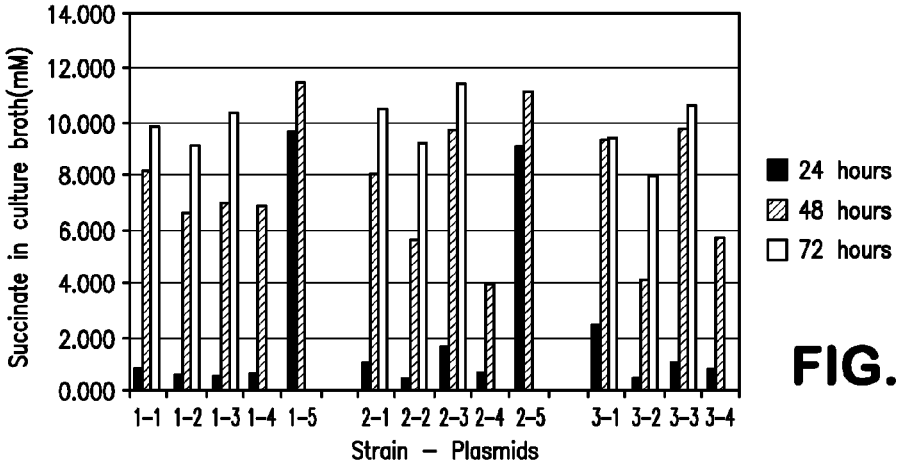


FIG. 3(b)

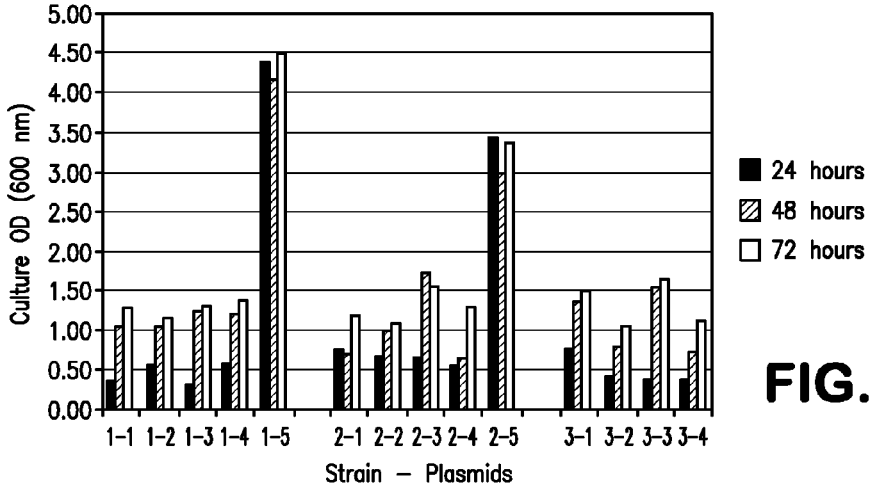


FIG. 3(c)

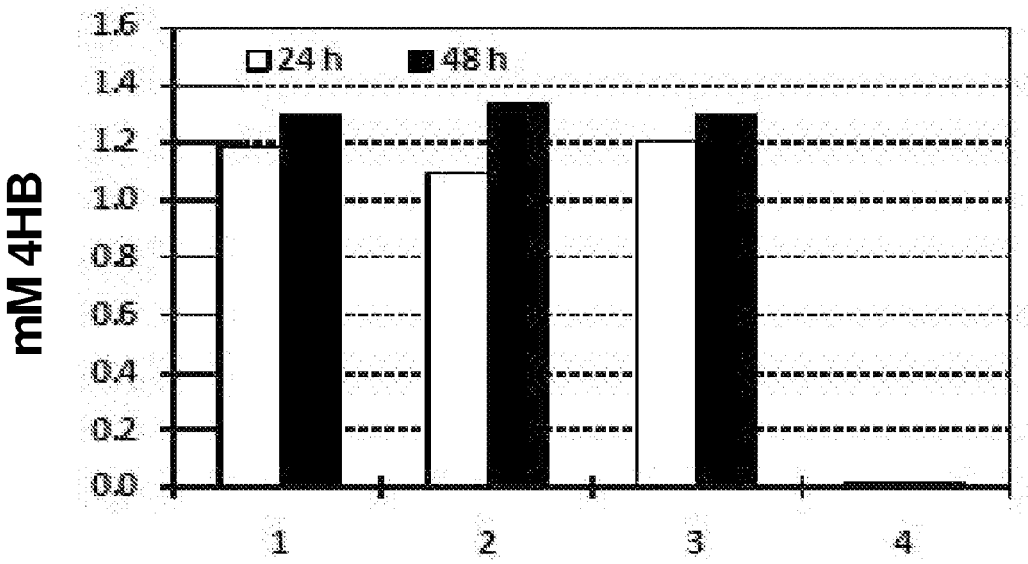


FIGURE 4

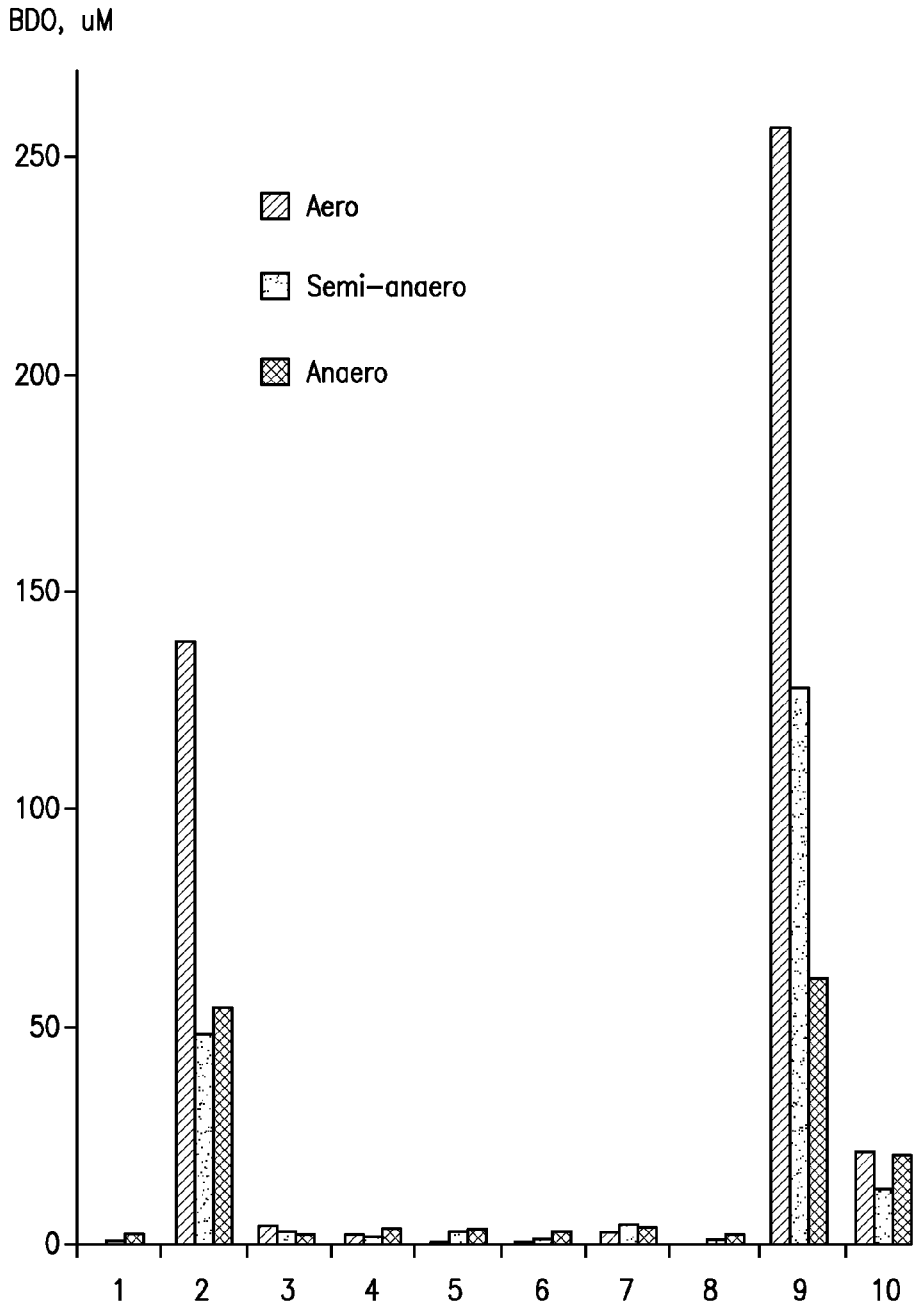

BDO, uM
250
200
150
100
50
0
Aero
Semi-anaero
Anaero
1
2
3
4
5
6
7
8
9
10

FIG. 5

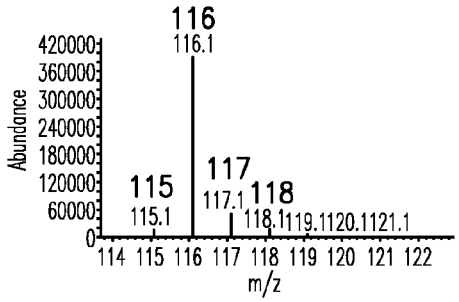


FIG. 6(a)

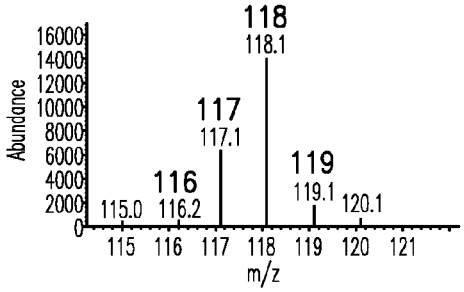


FIG. 6(b)

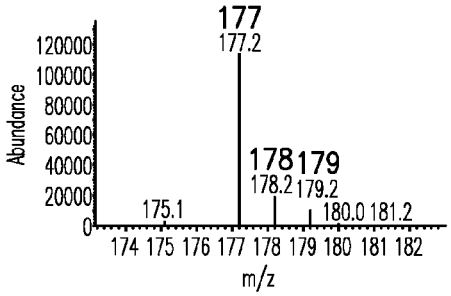


FIG. 6(c)

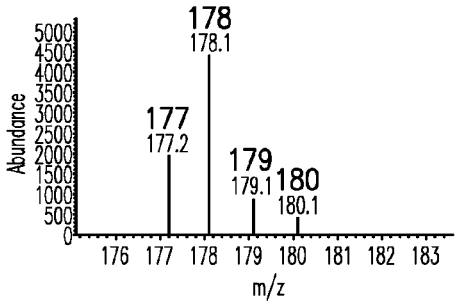


FIG. 6(d)

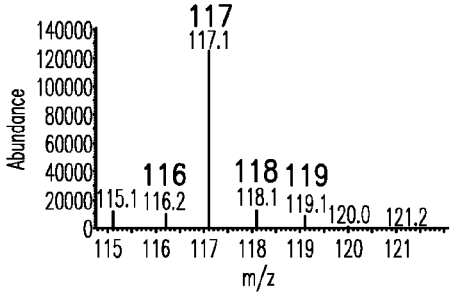


FIG. 6(e)

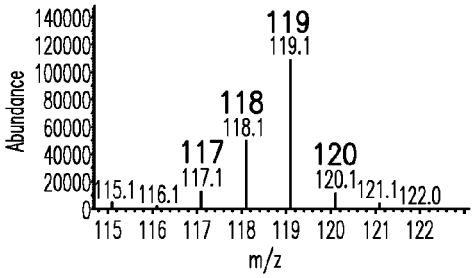


FIG. 6(f)

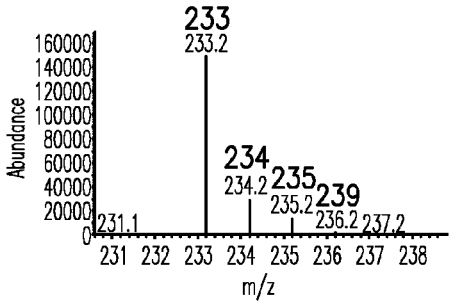


FIG. 6(g)

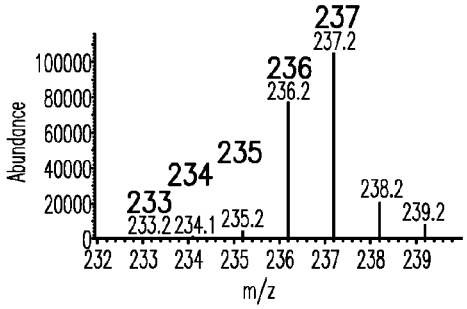


FIG. 6(h)

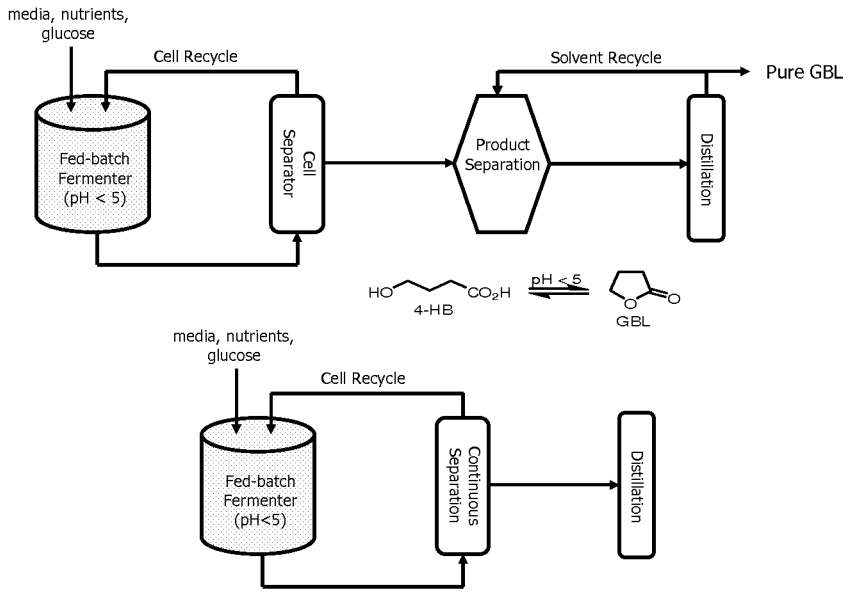

media, nutrients, glucose
Cell Recycle
Fed-batch Fermenter (pH < 5)
Cell Separator
Product Separation
Solvent Recycle
Pure GBL
Distillation
HO
CO2H
4-HB
pH < 5
GBL
media, nutrients, glucose
Cell Recycle
Fed-batch Fermenter (pH<5)
Continuous Separation
Distillation

FIGURE 7

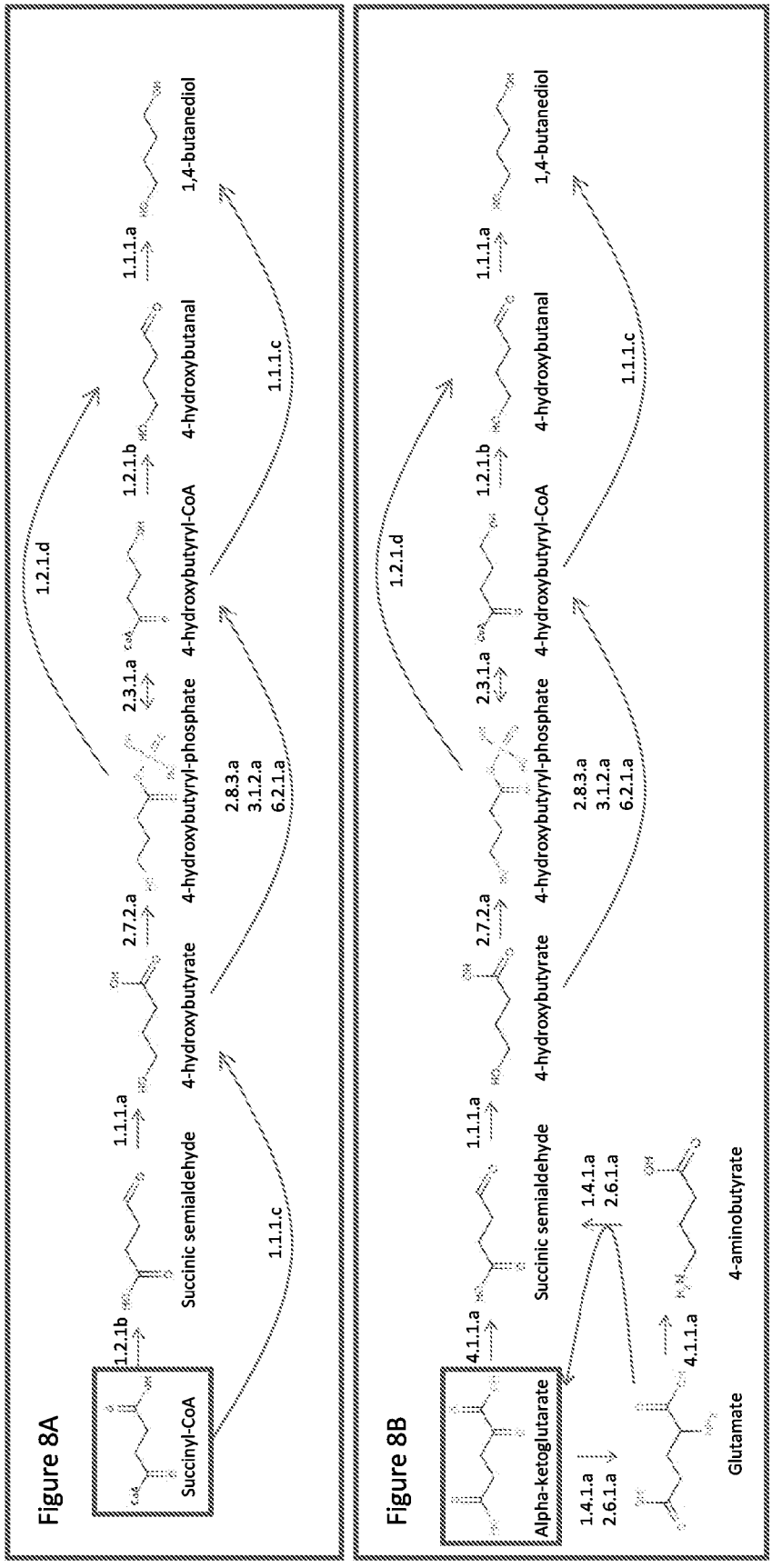


Figure 8A
Succinyl-CoA
1.2.1.b
Succinic semialdehyde
1.1.1.a
4-hydroxybutyrate
2.7.2.a
4-hydroxybutyryl-phosphate
2.3.1.a
4-hydroxybutyryl-CoA
1.2.1.b
4-hydroxybutanal
1.1.1.a
1,4-butanediol
1.2.1.d
1.1.1.c
2.8.3.a
3.1.2.a
6.2.1.a
Figure 8B
Alpha-ketoglutarate
4.1.1.a
Succinic semialdehyde
1.1.1.a
4-hydroxybutyrate
2.7.2.a
4-hydroxybutyryl-phosphate
2.3.1.a
4-hydroxybutyryl-CoA
1.2.1.b
4-hydroxybutanal
1.1.1.a
1,4-butanediol
1.2.1.d
1.1.1.c
2.8.3.a
3.1.2.a
6.2.1.a
1.4.1.a
2.6.1.a
Glutamate
4.1.1.a
4-aminobutyrate

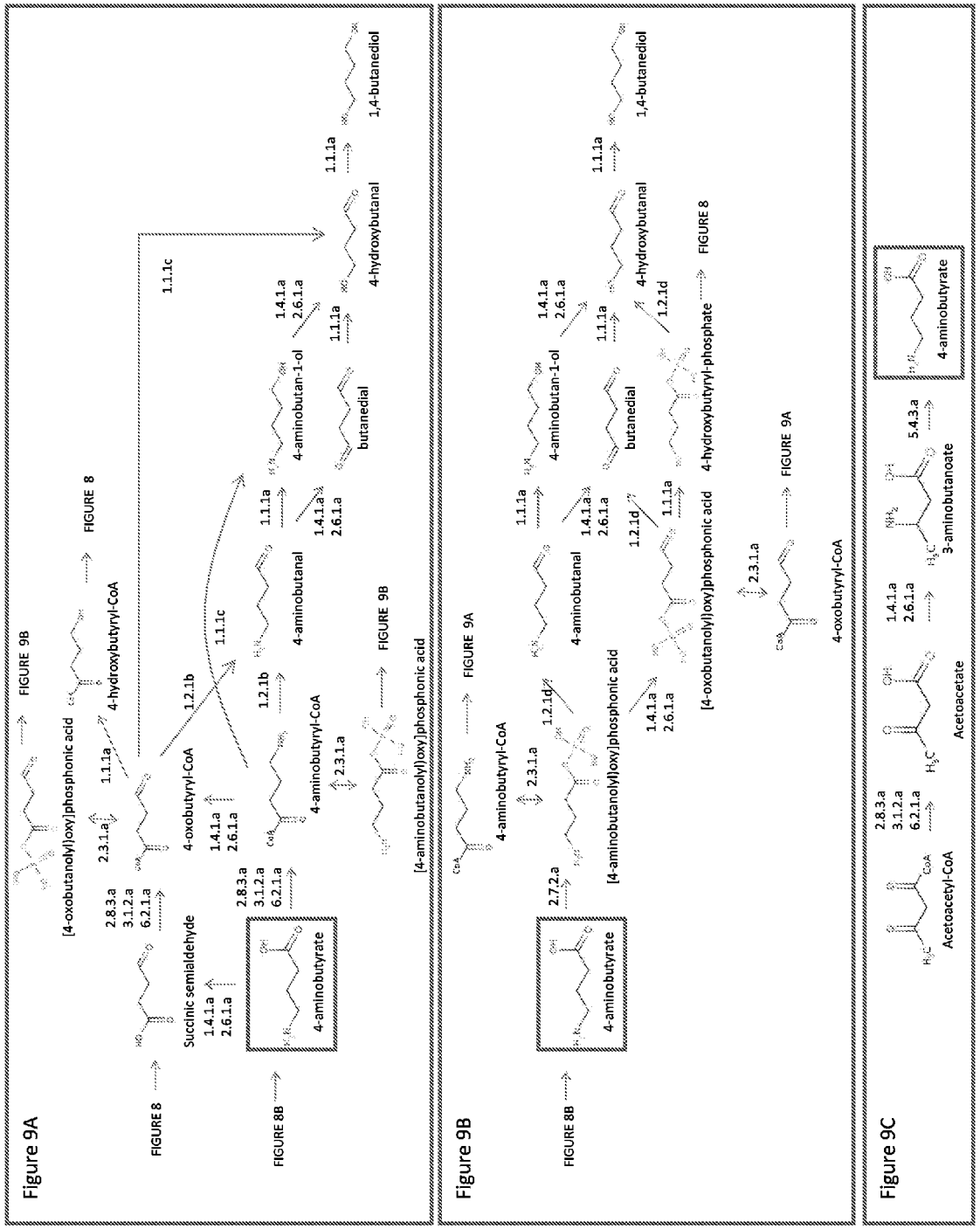


Figure 9A
FIGURE 9B
[4-oxobutanolyl)oxy]phosphonic acid
FIGURE 8
2.3.1.a
1.1.1a
4-hydroxybutyryl-CoA
2.8.3.a
3.1.2.a
6.2.1.a
1.1.1c
FIGURE 8
Succinic semialdehyde
4-oxobutyryl-CoA
1.2.1b
1.4.1.a
2.6.1.a
2.8.3.a
3.1.2.a
6.2.1.a
1.1.1c
1.2.1b
1.1.1a
4-aminobutan-1-ol
1.4.1.a
2.6.1.a
1.1.1a
4-hydroxybutanal
1.1.1a
1,4-butanediol
FIGURE 8B
4-aminobutyrate
4-aminobutyryl-CoA
2.3.1.a
4-aminobutanal
1.4.1.a
2.6.1.a
butanedial
FIGURE 9B
[4-aminobutanolyl)oxy]phosphonic acid
Figure 9B
FIGURE 9A
4-aminobutyryl-CoA
1.1.1a
4-aminobutan-1-ol
1.4.1.a
2.6.1.a
2.3.1.a
1.2.1d
4-aminobutanal
1.4.1.a
2.6.1.a
butanedial
1.1.1a
4-hydroxybutanal
1.1.1a
1,4-butanediol
2.7.2.a
FIGURE 8B
4-aminobutyrate
[4-aminobutanolyl)oxy]phosphonic acid
1.4.1.a
2.6.1.a
1.2.1d
[4-oxobutanolyl)oxy]phosphonic acid
4-hydroxybutyryl-phosphate
1.2.1d
FIGURE 8
2.3.1.a
FIGURE 9A
4-oxobutyryl-CoA
Figure 9C
2.8.3.a
3.1.2.a
6.2.1.a
Acetoacetyl-CoA
Acetoacetate
1.4.1.a
2.6.1.a
5.4.3.a
3-aminobutanoate
4-aminobutyrate

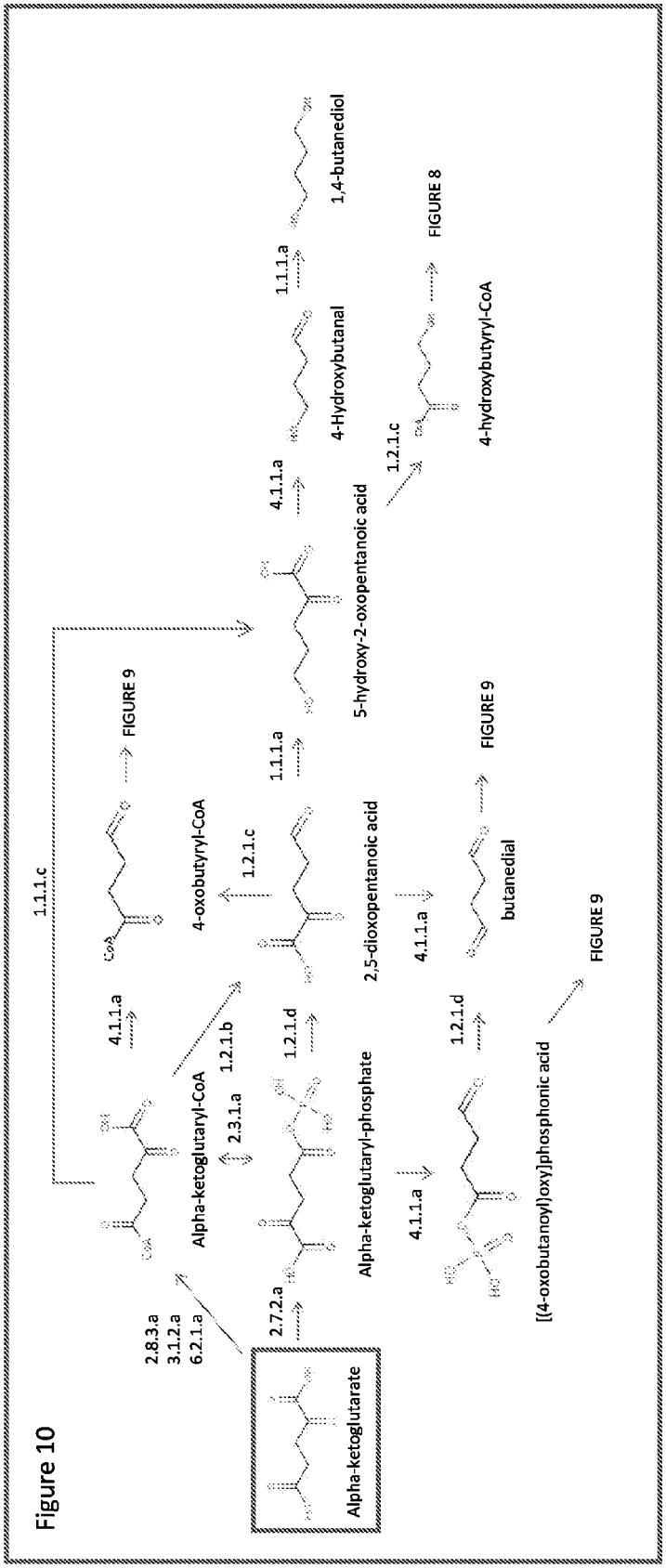


Figure 10
Alpha-ketoglutarate
2.8.3.a
3.1.2.a
6.2.1.a
2.7.2.a
Alpha-ketoglutaryl-phosphate
4.1.1.a
[(4-oxobutanoyl)oxy]phosphonic acid
1.2.1.d
FIGURE 9
Alpha-ketoglutaryl-CoA
2.3.1.a
1.2.1.b
4-oxobutyryl-CoA
1.2.1.c
2,5-dioxopentanoic acid
4.1.1.a
butanedial
1.2.1.d
FIGURE 9
1.1.1.c
FIGURE 9
1.1.1.a
5-hydroxy-2-oxopentanoic acid
4.1.1.a
4-Hydroxybutanal
1.1.1.a
1,4-butanediol
1.2.1.c
4-hydroxybutyryl-CoA
FIGURE 8

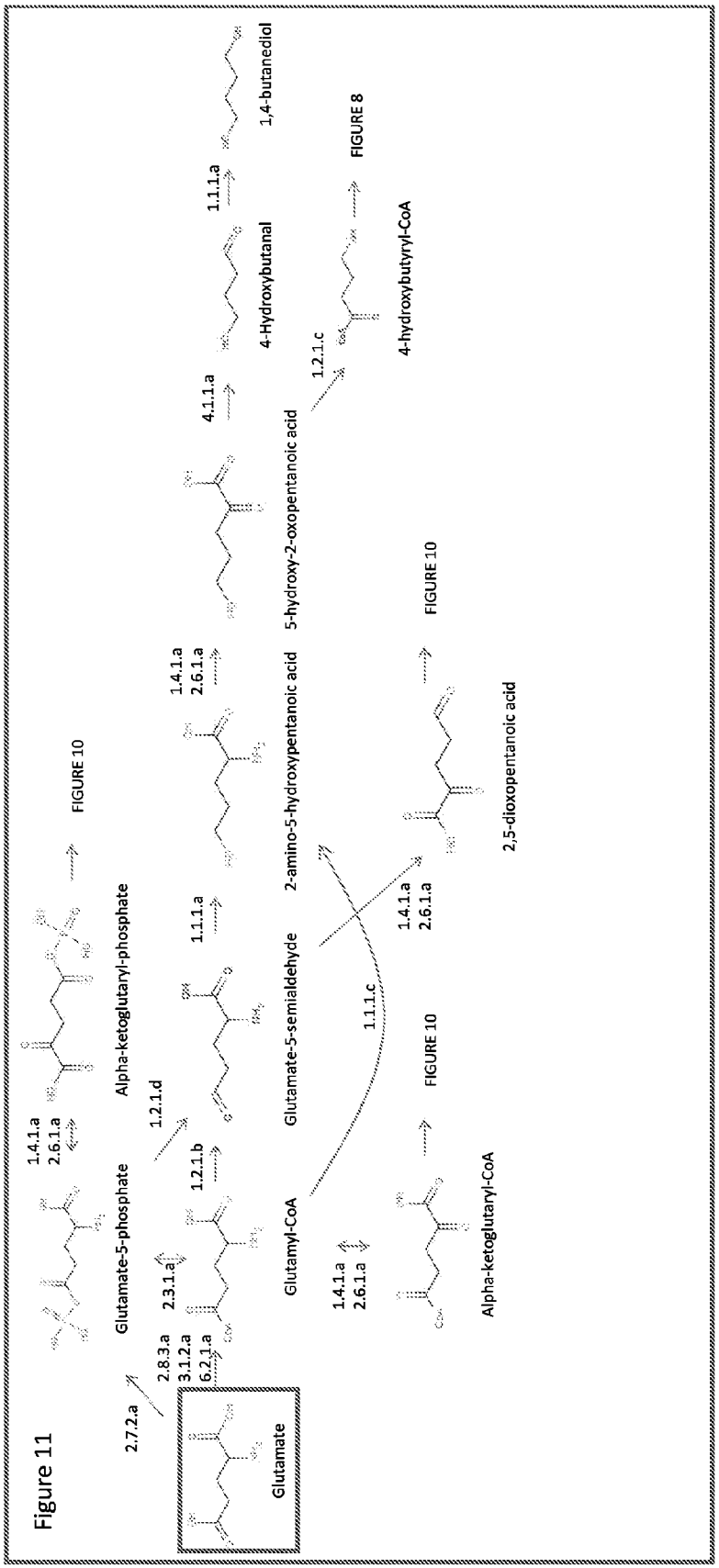


Figure 11
Glutamate
2.7.2.a
2.8.3.a
3.1.2.a
6.2.1.a
Glutamate-5-phosphate
1.4.1.a
2.6.1.a
Alpha-ketoglutaryl-phosphate
FIGURE 10
1.2.1.d
2.3.1.a
1.2.1.b
Glutamyl-CoA
Glutamate-5-semialdehyde
1.1.1.a
1.4.1.a
2.6.1.a
2-amino-5-hydroxypentanoic acid
1.4.1.a
2.6.1.a
1.1.1.c
FIGURE 10
Alpha-ketoglutaryl-CoA
2,5-dioxopentanoic acid
4.1.1.a
5-hydroxy-2-oxopentanoic acid
1.2.1.c
4-hydroxybutyryl-CoA
FIGURE 8
1.1.1.a
4-Hydroxybutanal
1,4-butanediol

Figure 12

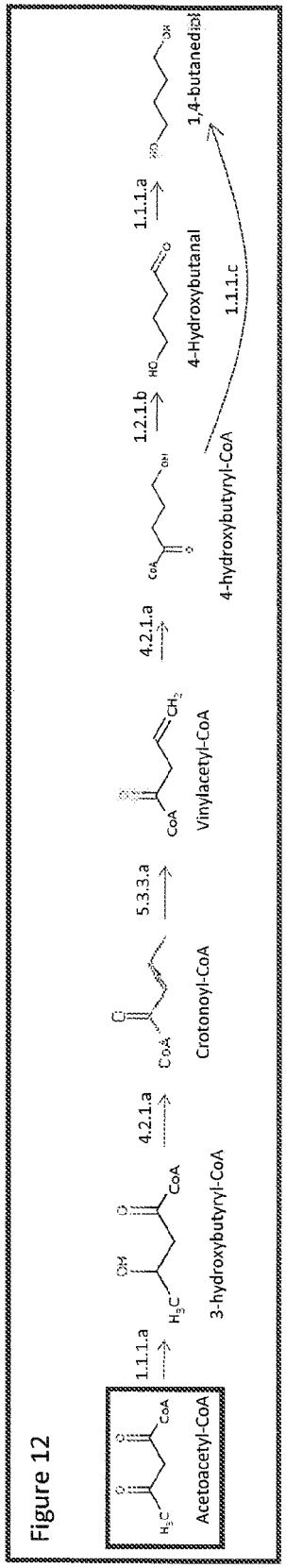
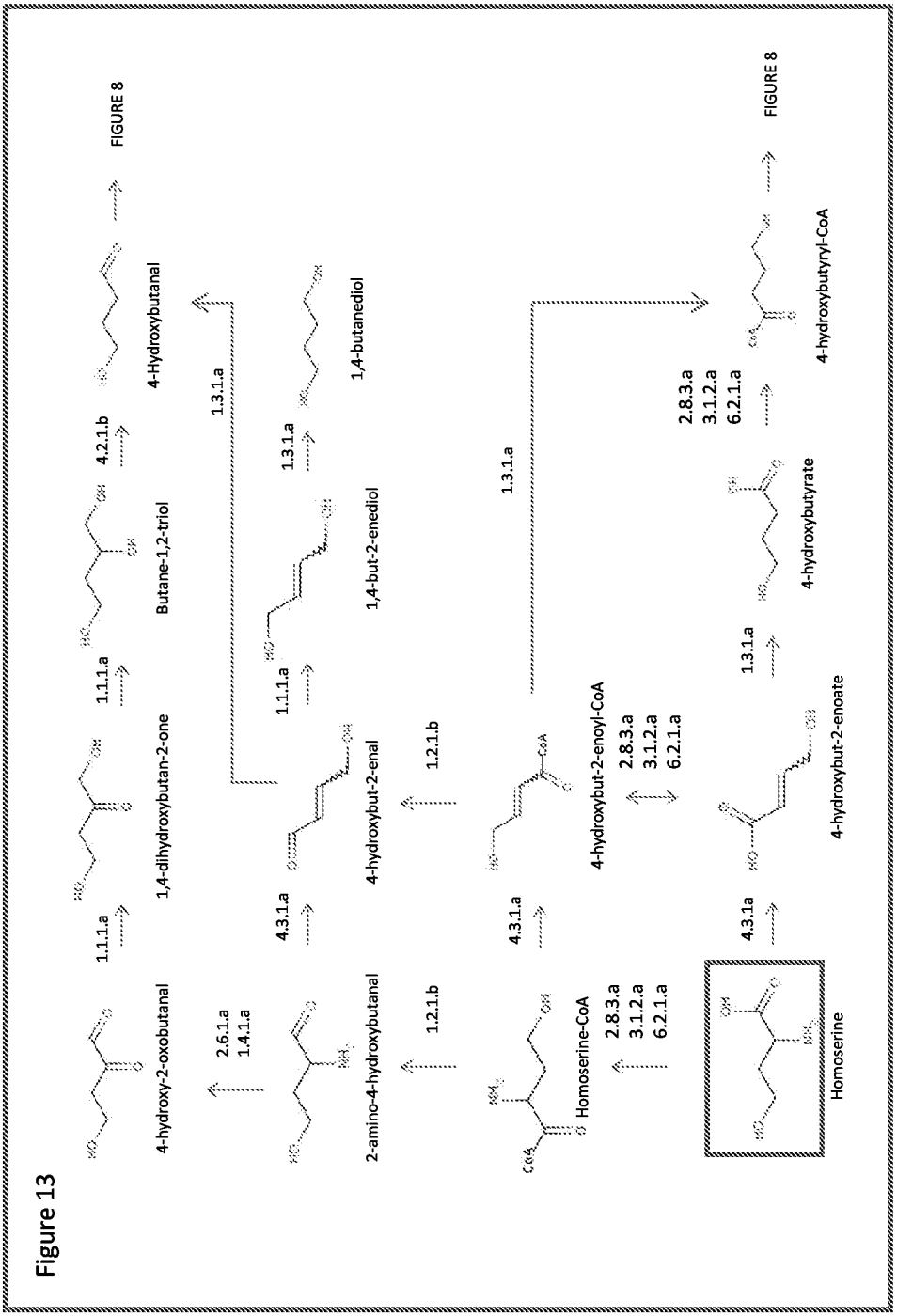

Acetoacetyl-CoA
1.1.1.a
3-hydroxybutyryl-CoA
4.2.1.a
Crotonoyl-CoA
5.3.3.a
Vinylacetyl-CoA
4.2.1.a
4-hydroxybutyryl-CoA
1.2.1.b
4-Hydroxybutanal
1.1.1.a
1,4-butanediol
1.1.1.c

Figure 13


4-hydroxy-2-oxobutanal
1.1.1.a
1,4-dihydroxybutan-2-one
1.1.1.a
Butane-1,2-triol
4.2.1.b
4-Hydroxybutanal
FIGURE 8
2.6.1.a
1.4.1.a
1.3.1.a
2-amino-4-hydroxybutanal
4.3.1.a
4-hydroxybut-2-enal
1.1.1.a
1,4-but-2-enediol
1.3.1.a
1,4-butanediol
1.2.1.b
1.2.1.b
Homoserine-CoA
4.3.1.a
4-hydroxybut-2-enoyl-CoA
1.3.1.a
2.8.3.a
3.1.2.a
6.2.1.a
2.8.3.a
3.1.2.a
6.2.1.a
4.3.1a
1.3.1.a
4-hydroxybutyrate
6.2.1.a
4-hydroxybutyryl-CoA
FIGURE 8
Homoserine
4-hydroxybut-2-enoate

A.

ATGAACTTACATGAATATCAGGCAAAACAACTTTTTGCCCGCTATGGCTTACCAGCACCGGTGGGTTATG
CCTGTACTACTCCGCGCGAAGCAGAAGAAGCCGCTTCAAAAATCGGTGCCGGTCCGTGGGTAGTGAAAT
GTCAGGTTCACGCTGGTGGCCGCGGTAAAGCGGGCGGTGTGAAAGTTGTAAACAGCAAAGAAGACATC
CGTGCTTTTGCAGAAAACTGGCTGGGCAAGCGTCTGGTAACGTATCAAACAGATGCCAATGGCCAACCG
GTTAACCAGATTCTGGTTGAAGCAGCGACCGATATCGCTAAAGAGCTGTATCTCGGTGCCGTTGTTGAC
CGTAGTTCCCGTCGTGTGGTCTTTATGGCCTCCACCGAAGGCGGCGTGGAAATCGAAAAAGTGGCGGA
AGAAACTCCGCACCTGATCCATAAAGTTGCGCTTGATCCGCTGACTGGCCCGATGCCGTATCAGGGACG
CGAGCTGGCGTTCAAACTGGGTCTGGAAGGTAAACTGGTTCAGCAGTTCACCAAAATCTTCATGGGCCT
GGCGACCATTTTCCTGGAGCGCGACCTGGCGTTGATCGAAATCAACCCGCTGGTCATCACCAAACAGGG
CGATCTGATTTGCCTCGACGGCAAACTGGGCGCTGACGGCAACGCACTGTTCCGCCAGCCTGATCTGCG
CGAAATGCGTGACCAGTCGCAGGAAGATCCGCGTGAAGCACAGGCTGCACAGTGGGAACTGAACTACG
TTGCGCTGGACGGTAACATCGGTTGTATGGTTAACGGCGCAGGTCTGGCGATGGGTACGATGGACATC
GTTAAACTGCACGGCGGCGAACCGGCTAACTTCCTTGACGTTGGCGGCGGCGCAACCAAAGAACGTGT
AACCGAAGCGTTCAAAATCATCCTCTCTGACGACAAAGTGAAAGCCGTTCTGGTTAACATCTTCGGCGGT
ATCGTTCGTTGCGACCTGATCGCTGACGGTATCATCGGCGCGGTAGCAGAAGTGGGTGTTAACGTACCG
GTCGTGGTACGTCTGGAAGGTAACAACGCCGAACTCGGCGCGAAGAAACTGGCTGACAGCGGCCTGAA
TATTATTGCAGCAAAAGGTCTGACGGATGCAGCTCAGCAGGTTGTTGCCGCAGTGGAGGGGAAATAAT
GTCCATTTTAATCGATAAAAACACCAAGGTTATCTGCCAGGGCTTTACCGGTAGCCAGGGGACTTTCCAC
TCAGAACAGGCCATTGCATACGGCACTAAAATGGTTGGCGGCGTAACCCCAGGTAAAGGCGGCACCAC
CCACCTCGGCCTGCCGGTGTTCAACACCGTGCGTGAAGCCGTTGCTGCCACTGGCGCTACCGCTTCTGTT
ATCTACGTACCAGCACCGTTCTGCAAAGACTCCATTCTGGAAGCCATCGACGCAGGCATCAAACTGATTA
TCACCATCACTGAAGGCATCCCGACGCTGGATATGCTGACCGTGAAAGTGAAGCTGGATGAAGCAGGC
GTTCGTATGATCGGCCCGAACTGCCCAGGCGTTATCACTCCGGGTGAATGCAAAATCGGTATCCAGCCT
GGTCACATTCACAAACCGGGTAAAGTGGGTATCGTTTCCCGTTCCGGTACACTGACCTATGAAGCGGTT
AAACAGACCACGGATTACGGTTTCGGTCAGTCGACCTGTGTCGGTATCGGCGGTGACCCGATCCCGGGC
TCTAACTTTATCGACATTCTCGAAATGTTCGAAAAAGATCCGCAGACCGAAGCGATCGTGATGATCGGT
GAGATCGGCGGTAGCGCTGAAGAAGAAGCAGCTGCGTACATCAAAGAGCACGTTACCAAGCCAGTTGT
GGGTTACATCGCTGGTGTGACTGCGCCGAAAGGCAAACGTATGGGCCACGCGGGTGCCATCATTGCCG
GTGGGAAAGGGACTGCGGATGAGAAATTCGCTGCTCTGGAAGCCGCAGGCGTGAAAACCGTTCGCAGC
CTGGCGGATATCGGTGAAGCACTGAAAACTGTTCTGAAATAA

B.

MNLHEYQAKQLFARYGLPAPVGYACTTPREAEEAASKIGAGPWVVKCQVHAGGRGKAGGVKVVNSKEDIR
AFAENWLGKRLVTYQTDANGQPVNQILVEAATDIAKELYLGAVVDRSSRRVVFMASTEGGVEIEKVAEETPH
LIHKVALDPLTGPMPYQGRELAFKLGLEGKLVQQFTKIFMGLATIFLERDLALIEINPLVITKQGDLICLDGKLGA
DGNALFRQPDLREMRDQSQEDPREAQAAQWELNYVALDGNIGCMVNGAGLAMGTMDIVKLHGGEPAN
FLDVGGGATKERVTEAFKIILSDDKVKAVLVNIFGGIVRCDLIADGIIGAVAEVGVNVPVVVRLEGNNAELGAK
KLADSGLNIIAAKGLTDAAQQVVAAVEGK

C.

MSILIDKNTKVICQGFTGSQGTFHSEQAIAYGTKMVGGVTPGKGGTTHLGLPVFNTVREAVAATGATASVIY
VPAPFCKDSILEAIDAGIKLIITITEGIPTLDMLTVKVKLDEAGVRMIGPNCPGVITPGECKIGIQPGHIHKPGKV
GIVSRSGTLTYEAVKQTTDYGFGQSTCVGIGGDPIPGSNFIDILEMFEKDPQTEAIVMIGEIGGSAEEEAAAYIK
EHVTKPVVGYIAGVTAPKGKRMGHAGAIIAGGKGTADEKFAALEAAGVKTVRSLADIGEALKTVLK

FIGURE 14

A.

ATGGCCAACATAAGTTCACCATTCGGGCAAAACGAATGGCTGGTTGAAGAGATGTACCGCAAGTTCCGC
GACGACCCCTCCTCGGTCGATCCCAGCTGGCACGAGTTCCTGGTTGACTACAGCCCCGAACCCACCTCCC
AACCAGCTGCCGAACCAACCCGGGTTACCTCGCCACTCGTTGCCGAGCGGGCCGCTGCGGCCGCCCCGC
AGGCACCCCCCAAGCCGGCCGACACCGCGGCCGCGGGCAACGGCGTGGTCGCCGCACTGGCCGCCAAA
ACTGCCGTTCCCCCGCCAGCCGAAGGTGACGAGGTAGCGGTGCTGCGCGGCGCCGCCGCGGCCGTCGT
CAAGAACATGTCCGCGTCGTTGGAGGTGCCGACGGCGACCAGCGTCCGGGCGGTCCCGGCCAAGCTAC
TGATCGACAACCGGATCGTCATCAACAACCAGTTGAAGCGGACCCGCGGCGGCAAGATCTCGTTCACGC
ATTTGCTGGGCTACGCCCTGGTGCAGGCGGTGAAGAAATTCCCGAACATGAACCGGCACTACACCGAA
GTCGACGGCAAGCCCACCGCGGTCACGCCGGCGCACACCAATCTCGGCCTGGCGATCGACCTGCAAGG
CAAGGACGGGAAGCGTTCCCTGGTGGTGGCCGGCATCAAGCGGTGCGAGACCATGCGATTCGCGCAGT
TCGTCACGGCCTACGAAGACATCGTACGCCGGGCCCGCGACGGCAAGCTGACCACTGAAGACTTTGCCG
GCGTGACGATTTCGCTGACCAATCCCGGAACCATCGGCACCGTGCATTCGGTGCCGCGGCTGATGCCCG
GCCAGGGCGCCATCATCGGCGTGGGCGCCATGGAATACCCCGCCGAGTTTCAAGGCGCCAGCGAGGAA
CGCATCGCCGAGCTGGGCATCGGCAAATTGATCACTTTGACCTCCACCTACGACCACCGCATCATCCAGG
GCGCGGAATCGGGCGACTTCCTGCGCACCATCCACGAGTTGCTGCTCTCGGATGGCTTCTGGGACGAGG
TCTTCCGCGAACTGAGCATCCCATATCTGCCGGTGCGCTGGAGCACCGACAACCCCGACTCGATCGTCG
ACAAGAACGCTCGCGTCATGAACTTGATCGCGGCCTACCGCAACCGCGGCCATCTGATGGCCGATACCG
ACCCGCTGCGGTTGGACAAAGCTCGGTTCCGCAGTCACCCCGACCTCGAAGTGCTGACCCACGGCCTGA
CGCTGTGGGATCTCGATCGGGTGTTCAAGGTCGACGGCTTTGCCGGTGCGCAGTACAAGAAACTGCGC
GACGTGCTGGGCTTGCTGCGCGATGCCTACTGCCGCCACATCGGCGTGGAGTACGCCCATATCCTCGAC
CCCGAACAAAAGGAGTGGCTCGAACAACGGGTCGAGACCAAGCACGTCAAACCCACTGTGGCCCAACA
GAAATACATCCTCAGCAAGCTCAACGCCGCCGAGGCCTTTGAAACGTTCCTACAGACCAAGTACGTCGG
CCAGAAGCGGTTCTCGCTGGAAGGCGCCGAAAGCGTGATCCCGATGATGGACGCGGCGATCGACCAGT
GCGCTGAGCACGGCCTCGACGAGGTGGTCATCGGGATGCCGCACCGGGGCCGGCTCAACGTGCTGGCC
AACATCGTCGGCAAGCCGTACTCGCAGATCTTCACCGAGTTCGAGGGCAACCTGAATCCGTCGCAGGCG
CACGGCTCCGGTGACGTCAAGTACCACCTGGGCGCCACCGGGCTGTACCTGCAGATGTTCGGCGACAAC
GACATTCAGGTGTCGCTGACCGCCAACCCGTCGCATCTGGAGGCCGTCGACCCGGTGCTGGAGGGATT
GGTGCGGGCCAAGCAGGATCTGCTCGACCACGGAAGCATCGACAGCGACGGCCAACGGGCGTTCTCGG
TGGTGCCGCTGATGTTGCATGGCGATGCCGCGTTCGCCGGTCAGGGTGTGGTCGCCGAGACGCTGAAC
CTGGCGAATCTGCCGGGCTACCGCGTCGGCGGCACCATCCACATCATCGTCAACAACCAGATCGGCTTC
ACCACCGCGCCCGAGTATTCCAGGTCCAGCGAGTACTGCACCGACGTCGCAAAGATGATCGGGGCACC
GATCTTTCACGTCAACGGCGACGACCCGGAGGCGTGTGTCTGGGTGGCGCGGTTGGCGGTGGACTTCC
GACAACGGTTCAAGAAGGACGTCGTCATCGACATGCTGTGCTACCGCCGCCGCGGGCACAACGAGGGT
GACGACCCGTCGATGACCAACCCCTACATGTACGACGTCGTCGACACCAAGCGCGGGGCCCGCAAAAG
CTACACCGAAGCCCTGATCGGACGTGGCGACATCTCGATGAAGGAGGCCGAGGACGCGCTGCGCGACT
ACCAGGGCCAGCTGGAACGGGTGTTCAACGAAGTGCGCGAGCTGGAGAAGCACGGTGTGCAGCCGAG
CGAGTCGGTCGAGTCCGACCAGATGATTCCCGCGGGGCTGGCCACTGCGGTGGACAAGTCGCTGCTGG
CCCGGATCGGCGATGCGTTCCTCGCCTTGCCGAACGGCTTCACCGCGCACCCGCGAGTCCAACCGGTGC
TGGAGAAGCGCCGGGAGATGGCCTATGAAGGCAAGATCGACTGGGCCTTTGGCGAGCTGCTGGCGCT
GGGCTCGCTGGTGGCCGAAGGCAAGCTGGTGCGCTTGTCGGGGCAGGACAGCCGCCGCGGCACCTTCT
CCCAGCGGCATTCGGTTCTCATCGACCGCCACACTGGCGAGGAGTTCACACCACTGCAGCTGCTGGCGA
CCAACTCCGACGGCAGCCCGACCGGCGGAAAGTTCCTGGTCTACGACTCGCCACTGTCGGAGTACGCCG
CCGTCGGCTTCGAGTACGGCTACACTGTGGGCAATCCGGACGCCGTGGTGCTCTGGGAGGCGCAGTTC

FIGURE 15

GGCGACTTCGTCAACGGCGCACAGTCGATCATCGACGAGTTCATCAGCTCCGGTGAGGCCAAGTGGGG
CCAATTGTCCAACGTCGTGCTGCTGTTACCGCACGGGCACGAGGGGCAGGGACCCGACCACACTTCTGC
CCGGATCGAACGCTTCTTGCAGTTGTGGGCGGAAGGTTCGATGACCATCGCGATGCCGTCGACTCCGTC
GAACTACTTCCACCTGCTACGCCGGCATGCCCTGGACGGCATCCAACGCCCGCTGATCGTGTTCACGCCC
AAGTCGATGTTGCGTCACAAGGCCGCCGTCAGCGAAATCAAGGACTTCACCGAGATCAAGTTCCGCTCA
GTGCTGGAGGAACCCACCTATGAGGACGGCATCGGAGACCGCAACAAGGTCAGCCGGATCCTGCTGAC
CAGTGGCAAGCTGTATTACGAGCTGGCCGCCCGCAAGGCCAAGGACAACCGCAATGACCTCGCGATCG
TGCGGCTTGAACAGCTCGCCCCGCTGCCCAGGCGTCGACTGCGTGAAACGCTGGACCGCTACGAGAAC
GTCAAGGAGTTCTTCTGGGTCCAAGAGGAACCGGCCAACCAGGGTGCGTGGCCGCGATTCGGGCTCGA
ACTACCCGAGCTGCTGCCTGACAAGTTGGCCGGGATCAAGCGAATCTCGCGCCGGGCGATGTCAGCCCC
GTCGTCAGGCTCGTCGAAGGTGCACGCCGTCGAACAGCAGGAGATCCTCGACGAGGCGTTCGGCTAA

B.

MANISSPFGQNEWLVEEMYRKFRDDPSSVDPSWHEFLVDYSPEPTSQPAAEPTRVTSPLVAERAAAAAPQA
PPKPADTAAAGNGVVAALAAKTAVPPPAEGDEVAVLRGAAAAVVKNMSASLEVPTATSVRAVPAKLLIDNR
IVINNQLKRTRGGKISFTHLLGYALVQAVKKFPNMNRHYTEVDGKPTAVTPAHTNLGLAIDLQGKDGKRSLV
VAGIKRCETMRFAQFVTAYEDIVRRARDGKLTTEDFAGVTISLTNPGTIGTVHSVPRLMPGQGAIIGVGAME
YPAEFQGASEERIAELGIGKLITLTSTYDHRIIQGAESGDFLRTIHELLLSDGFWDEVFRELSIPYLPVRWSTDNP
DSIVDKNARVMNLIAAYRNRGHLMADTDPLRLDKARFRSHPDLEVLTHGLTLWDLDRVFKVDGFAGAQYKK
LRDVLGLLRDAYCRHIGVEYAHILDPEQKEWLEQRVETKHVKPTVAQQKYILSKLNAAEAFETFLQTKYVGQK
RFSLEGAESVIPMMDAAIDQCAEHGLDEVVIGMPHRGRLNVLANIVGKPYSQIFTEFEGNLNPSQAHGSGD
VKYHLGATGLYLQMFGDNDIQVSLTANPSHLEAVDPVLEGLVRAKQDLLDHGSIDSDGQRAFSVVPLMLHG
DAAFAGQGVVAETLNLANLPGYRVGGTIHIIVNNQIGFTTAPEYSRSSEYCTDVAKMIGAPIFHVNGDDPEAC
VWVARLAVDFRQRFKKDVVIDMLCYRRRGHNEGDDPSMTNPYMYDVVDTKRGARKSYTEALIGRGDISM
KEAEDALRDYQGQLERVFNEVRELEKHGVQPSESVESDQMIPAGLATAVDKSLLARIGDAFLALPNGFTAHP
RVQPVLEKRREMAYEGKIDWAFGELLALGSLVAEGKLVRLSGQDSRRGTFSQRHSVLIDRHTGEEFTPLQLLA
TNSDGSPTGGKFLVYDSPLSEYAAVGFEYGYTVGNPDAVVLWEAQFGDFVNGAQSIIDEFISSGEAKWGQLS
NVVLLLPHGHEGQGPDHTSARIERFLQLWAEGSMTIAMPSTPSNYFHLLRRHALDGIQRPLIVFTPKSMLRH
KAAVSEIKDFTEIKFRSVLEEPTYEDGIGDRNKVSRILLTSGKLYYELAARKAKDNRNDLAIVRLEQLAPLPRRRL
RETLDRYENVKEFFWVQEEPANQGAWPRFGLELPELLPDKLAGIKRISRRAMSAPSSGSSKVHAVEQQEILDE
AFG

FIGURE 15 (cont'd)

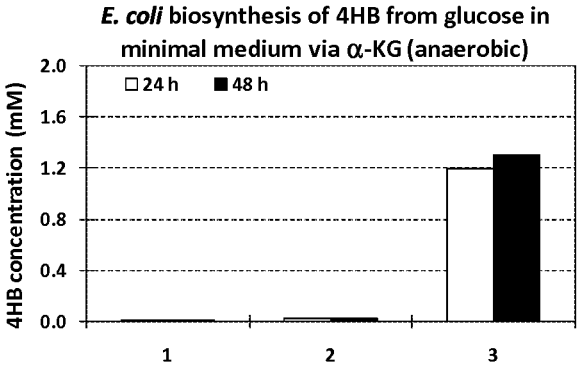


FIGURE 16

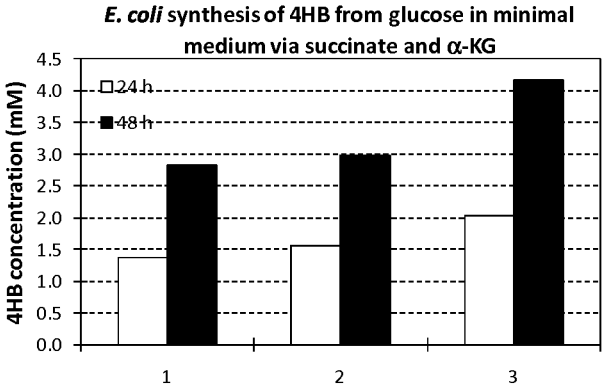


FIGURE 17

A.

ATGGAAATCAAAGAAATGGTGAGCCTTGCACGCAAGGCTCAGAAGGAGTATCAAGCTACCCATAACCA
AGAAGCAGTTGACAACATTTGCCGAGCTGCAGCAAAAGTTATTTATGAAAATGCAGCTATTCTGGCTCG
CGAAGCAGTAGACGAAACCGGCATGGGCGTTTACGAACACAAAGTGGCCAAGAATCAAGGCAAATCCA
AAGGTGTTTGGTACAACCTCCACAATAAAAAATCGATTGGTATCCTCAATATAGACGAGCGTACCGGTAT
GATCGAGATTGCAAAGCCTATCGGAGTTGTAGGAGCCGTAACGCCGACGACCAACCCGATCGTTACTCC
GATGAGCAATATCATCTTTGCTCTTAAGACCTGCAATGCCATCATTATTGCCCCCCACCCCAGATCCAAAA
AATGCTCTGCACACGCAGTTCGTCTGATCAAAGAAGCTATCGCTCCGTTCAACGTACCGGAAGGTATGG
TTCAGATCATCGAAGAACCCAGCATCGAGAAGACGCAGGAACTCATGGGCGCCGTAGACGTAGTAGTT
GCTACGGGTGGTATGGGCATGGTGAAGTCTGCATATTCTTCAGGAAAGCCTTCTTTCGGTGTTGGAGCC
GGTAACGTTCAGGTGATCGTGGATAGCAACATCGATTTCGAAGCTGCTGCAGAAAAAATCATCACCGGT
CGTGCTTTCGACAACGGTATCATCTGCTCAGGCGAACAGAGCATCATCTACAACGAGGCTGACAAGGAA
GCAGTTTTCACAGCATTCCGCAACCACGGTGCATATTTCTGTGACGAAGCCGAAGGAGATCGGGCTCGT
GCAGCTATCTTCGAAAATGGAGCCATCGCGAAAGATGTAGTAGGTCAGAGCGTTGCCTTCATTGCCAAG
AAAGCAAACATCAATATCCCCGAGGGTACCCGTATTCTCGTTGTTGAAGCTCGCGGCGTAGGAGCAGAA
GACGTTATCTGTAAGGAAAAGATGTGTCCCGTAATGTGCGCCCTCAGCTACAAGCACTTCGAAGAAGGT
GTAGAAATCGCACGTACGAACCTCGCCAACGAAGGTAACGGCCACACCTGTGCTATCCACTCCAACAAT
CAGGCACACATCATCCTCGCAGGATCAGAGCTGACGGTATCTCGTATCGTAGTGAATGCTCCGAGTGCC
ACTACAGCAGGCGGTCACATCCAAAACGGTCTTGCCGTAACCAATACGCTCGGATGCGGATCATGGGGT
AATAACTCTATCTCCGAGAACTTCACTTACAAGCACCTCCTCAACATTTCACGCATCGCACCGTTGAATTC
AAGCATTCACATCCCCGATGACAAAGAAATCTGGGAACTCTAA

B.

MEIKEMVSLARKAQKEYQATHNQEAVDNICRAAAKVIYENAAILAREAVDETGMGVYEHKVAKNQGKSKG
VWYNLHNKKSIGILNIDERTGMIEIAKPIGVVGAVTPTTNPIVTPMSNIIFALKTCNAIIIAPHPRSKKCSAHAVR
LIKEAIAPFNVPEGMVQIIEEPSIEKTQELMGAVDVVVATGGMGMVKSAYSSGKPSFGVGAGNVQVIVDSNI
DFEAAAEKIITGRAFDNGIICSGEQSIIYNEADKEAVFTAFRNHGAYFCDEAEGDRARAAIFENGAIAKDVVGQ
SVAFIAKKANINIPEGTRILVVEARGVGAEDVICKEKMCPVMCALSYKHFEEGVEIARTNLANEGNGHTCAIHS
NNQAHIILAGSELTVSRIVVNAPSATTAGGHIQNGLAVTNTLGCGSWGNNSISENFTYKHLLNISRIAPLNSSI
HIPDDKEIWEL

FIGURE 18

A.

ATGCAACTTTTCAAACTCAAGAGTGTAACACATCACTTTGACACTTTTGCAGAATTTGCCAAGGAATTCTG
TCTTGGAGAACGCGACTTGGTAATTACCAACGAGTTCATCTATGAACCGTATATGAAGGCATGCCAGCTC
CCCTGCCATTTTGTTATGCAGGAGAAATATGGGCAAGGCGAGCCTTCTGACGAAATGATGAATAACATC
TTGGCAGACATCCGTAATATCCAGTTCGACCGCGTAATCGGTATCGGAGGAGGTACGGTTATTGACATC
TCTAAACTTTTCGTTCTGAAAGGATTAAATGATGTACTCGATGCATTCGACCGCAAAATACCTCTTATCAA
AGAGAAAGAACTGATCATTGTGCCCACAACATGCGGAACGGGTAGCGAGGTGACGAACATTTCTATCG
CAGAAATCAAAAGCCGTCACACCAAAATGGGATTGGCTGACGATGCCATTGTTGCAGACCATGCCATCA
TCATACCTGAACTTCTGAAGAGCTTGCCTTTCCACTTCTACGCATGCAGTGCAATCGATGCTCTTATCCAT
GCCATCGAGTCATACGTATCTCCTAAAGCCAGTCCATATTCTCGTCTGTTCAGTGAGGCGGCTTGGGACA
TTATCCTGGAAGTATTCAAGAAAATCGCCGAACACGGCCCTGAATACCGCTTCGAAAAGCTGGGAGAAA
TGATCATGGCCAGCAACTATGCCGGTATAGCCTTCGGAAATGCAGGAGTAGGAGCCGTCCACGCACTAT
CCTACCCGTTGGGAGGCAACTATCACGTGCCGCATGGAGAAGCAAACTATCAGTTCTTCACAGAGGTAT
TCAAAGTATACCAAAAGAAGAATCCTTTCGGCTATATAGTCGAACTCAACTGGAAGCTCTCCAAGATACT
GAACTGCCAGCCCGAATACGTATATCCGAAGCTGGATGAACTTCTCGGATGCCTTCTTACCAAGAAACCT
TTGCACGAATACGGCATGAAGGACGAAGAGGTAAGAGGCTTTGCGGAATCAGTGCTTAAGACACAGCA
AAGATTGCTCGCCAACAACTACGTAGAGCTTACTGTAGATGAGATCGAAGGTATCTACAGAAGACTCTA
CTAA

B.

MQLFKLKSVTHHFDTFAEFAKEFCLGERDLVITNEFIYEPYMKACQLPCHFVMQEKYGQGEPSDEMMNNIL
ADIRNIQFDRVIGIGGGTVIDISKLFVLKGLNDVLDAFDRKIPLIKEKELIIVPTTCGTGSEVTNISIAEIKSRHTKM
GLADDAIVADHAIIIPELLKSLPFHFYACSAIDALIHAIESYVSPKASPYSRLFSEAAWDIILEVFKKIAEHGPEYRFE
KLGEMIMASNYAGIAFGNAGVGAVHALSYPLGGNYHVPHGEANYQFFTEVFKVYQKKNPFGYIVELNWKLS
KILNCQPEYVYPKLDELLGCLLTKKPLHEYGMKDEEVRGFAESVLKTQQRLLANNYVELTVDEIEGIYRRLY

FIGURE 19

A.

ATGAAAGACGTATTAGCGGAATATGCCTCCCGAATTGTTTCGGCCGAAGAAGCCGTAAAACATATCAAA
AATGGAGAACGGGTAGCTTTGTCACATGCTGCCGGAGTTCCTCAGAGTTGTGTTGATGCACTGGTACAA
CAGGCCGACCTTTTCCAGAATGTCGAAATTTATCACATGCTTTGTCTCGGCGAAGGAAAATATATGGCAC
CTGAAATGGCCCCTCACTTCCGACACATAACCAATTTTGTAGGTGGTAATTCTCGTAAAGCAGTTGAGGA
AAATAGAGCCGACTTCATTCCGGTATTCTTTTATGAAGTGCCATCAATGATTCGCAAAGACATCCTTCACA
TAGATGTCGCCATCGTTCAGCTTTCAATGCCTGATGAGAATGGTTACTGTAGTTTTGGAGTATCTTGCGA
TTATAGCAAACCGGCAGCAGAAAGCGCTCATTTAGTTATAGGGGAAATCAACCGTCAAATGCCATATGT
ACATGGCGACAACTTGATTCACATATCGAAGTTGGATTACATCGTGATGGCAGACTACCCTATCTATTCT
CTTGCAAAGCCCAAAATCGGAGAAGTAGAAGAAGCTATCGGGCGTAATTGTGCCGAGCTTATTGAAGA
TGGTGCCACACTCCAACTCGGTATCGGCGCGATTCCTGATGCAGCCCTGTTATTCCTCAAGGACAAAAAA
GATCTGGGGATCCATACCGAGATGTTCTCCGATGGTGTTGTCGAATTAGTTCGCAGTGGAGTAATTACA
GGAAAGAAAAAGACACTTCACCCCGGAAAGATGGTCGCAACCTTCTTAATGGGAAGCGAAGACGTATA
TCATTTCATCGACAAAAATCCCGATGTAGAACTTTATCCGGTAGATTACGTCAATGATCCGCGAGTAATC
GCTCAAAATGATAATATGGTCAGCATCAATAGCTGTATCGAAATCGATCTTATGGGACAAGTCGTGTCC
GAATGTATAGGAAGCAAGCAATTCAGCGGAACCGGCGGTCAAGTAGATTATGTTCGTGGAGCAGCATG
GTCTAAAAACGGCAAAAGCATCATGGCAATTCCCTCAACAGCCAAAAACGGTACTGCATCTCGAATTGT
ACCTATAATTGCAGAGGGAGCTGCTGTAACAACCCTCCGCAACGAAGTCGATTACGTTGTAACCGAATA
CGGTATAGCACAACTCAAAGGAAAGAGTTTGCGCCAGCGAGCAGAAGCTCTTATTGCCATAGCCCACCC
GGATTTCAGAGAGGAACTAACGAAACATCTCCGCAAACGTTTCGGATAA

B.

MKDVLAEYASRIVSAEEAVKHIKNGERVALSHAAGVPQSCVDALVQQADLFQNVEIYHMLCLGEGKYMAPE
MAPHFRHITNFVGGNSRKAVEENRADFIPVFFYEVPSMIRKDILHIDVAIVQLSMPDENGYCSFGVSCDYSKP
AAESAHLVIGEINRQMPYVHGDNLIHISKLDYIVMADYPIYSLAKPKIGEVEEAIGRNCAELIEDGATLQLGIGAI
PDAALLFLKDKKDLGIHTEMFSDGVVELVRSGVITGKKKTLHPGKMVATFLMGSEDVYHFIDKNPDVELYPV
DYVNDPRVIAQNDNMVSINSCIEIDLMGQVVSECIGSKQFSGTGGQVDYVRGAAWSKNGKSIMAIPSTAKN
GTASRIVPIIAEGAAVTTLRNEVDYVVTEYGIAQLKGKSLRQRAEALIAIAHPDFREELTKHLRKRFG

FIGURE 20

A.

ATGATTAAGAGTTTTAATGAAATTATCATGAAGGTAAAGAGCAAAGAAATGAAAAAAGTTGCTGT
TGCTGTAGCACAAGACGAGCCAGTACTTGAAGCAGTAAGAGATGCTAAGAAAAATGGTATTGCAG
ATGCTATTCTTGTTGGAGACCATGACGAAATCGTGTCAATCGCGCTTAAAATAGGAATGGATGTA
AATGATTTTGAAATAGTAAACGAGCCTAACGTTAAGAAAGCTGCTTTAAAGGCAGTAGAGCTTGT
ATCAACTGGAAAAGCTGATATGGTAATGAAGGGACTTGTAAATACAGCAACTTTCTTAAGATCTG
TATTAAACAAAGAAGTTGGACTTAGAACAGGAAAAACTATGTCTCACGTTGCAGTATTTGAAACT
GAGAAATTTGATAGACTATTATTTTTAACAGATGTTGCTTTCAATACTTATCCTGAATTAAAGGA
AAAAATTGATATAGTAAACAATTCAGTTAAGGTTGCACATGCAATAGGAATTGAAAATCCAAAGG
TTGCTCCAATTTGTGCAGTTGAGGTTATAAACCCTAAAATGCCATCAACACTTGATGCAGCAATG
CTTTCAAAAATGAGTGACAGAGGACAAATTAAAGGTTGTGTAGTTGACGGACCTTTAGCACTTGA
TATAGCTTTATCAGAAGAAGCAGCACATCATAAGGGAGTAACAGGAGAAGTTGCTGGAAAAGCTG
ATATCTTCTTAATGCCAAACATAGAAACAGGAAATGTAATGTATAAGACTTTAACATATACAACT
GATTCAAAAAATGGAGGAATCTTAGTTGGAACTTCTGCACCAGTTGTTTTAACTTCAAGAGCTGA
CAGCCATGAAACAAAAATGAACTCTATAGCACTTGCAGCTTTAGTTGCAGGCAATAAATAA

B.

MIKSFNEIIMKVKSKEMKKVAVAVAQDEPVLEAVRDAKKNGIADAILVGDHDEIVSIALKIGMDV
NDFEIVNEPNVKKAALKAVELVSTGKADMVMKGLVNTATFLRSVLNKEVGLRTGKTMSHVAVFET
EKFDRLLFLTDVAFNTYPELKEKIDIVNNSVKVAHAIGIENPKVAPICAVEVINPKMPSTLDAAM
LSKMSDRGQIKGCVVDGPLALDIALSEEAAHHKGVTGEVAGKADIFLMPNIETGNVMYKTLTYTT
DSKNGGILVGTSAPVVLTSRADSHETKMNSIALAALVAGNK

FIGURE 21

A.

ATGTATAGATTACTAATAATCAATCCTGGCTCGACCTCAACTAAAATTGGTATTTATGACGATGA
AAAAGAGATATTTGAGAAGACTTTAAGACATTCAGCTGAAGAGATAGAAAAATATAACACTATAT
TTGATCAATTTCAATTCAGAAAGAATGTAATTTTAGATGCGTTAAAAGAAGCAAACATAGAAGTA
AGTTCTTTAAATGCTGTAGTTGGAAGAGGCGGACTCTTAAAGCCAATAGTAAGTGGAACTTATGC
AGTAAATCAAAAAATGCTTGAAGACCTTAAAGTAGGAGTTCAAGGTCAGCATGCGTCAAATCTTG
GTGGAATTATTGCAAATGAAATAGCAAAAGAAATAAATGTTCCAGCATACATAGTTGATCCAGTT
GTTGTGGATGAGCTTGATGAAGTTTCAAGAATATCAGGAATGGCTGACATTCCAAGAAAAAGTAT
ATTCCATGCATTAAATCAAAAAGCAGTTGCTAGAAGATATGCAAAAGAAGTTGGAAAAAAATACG
AAGATCTTAATTTAATCGTAGTCCACATGGGTGGAGGTACTTCAGTAGGTACTCATAAAGATGGT
AGAGTAATAGAAGTTAATAATACACTTGATGGAGAAGGTCCATTCTCACCAGAAAGAAGTGGTGG
AGTTCCAATAGGAGATCTTGTAAGATTGTGCTTCAGCAACAAATATACTTATGAAGAAGTAATGA
AAAAGATAAACGGCAAAGGCGGAGTTGTTAGTTACTTAAATACTATCGATTTTAAGGCTGTAGTT
GATAAAGCTCTTGAAGGAGATAAGAAATGTGCACTTATATATGAAGCTTTCACATTCCAGGTAGC
AAAAGAGATAGGAAAATGTTCAACCGTTTTAAAAGGAAATGTAGATGCAATAATCTTAACAGGCG
GAATTGCGTACAACGAGCATGTATGTAATGCCATAGAGGATAGAGTAAAATTCATAGCACCTGTA
GTTAGATATGGTGGAGAAGATGAACTTCTTGCACTTGCAGAAGGTGGACTTAGAGTTTTAAGAGG
AGAAGAAAAAGCTAAGGAATACAAATAA

B.

MYRLLIINPGSTSTKIGIYDDEKEIFEKTLRHSAEEIEKYNTIFDQFQFRKNVILDALKEANIEV
SSLNAVVGRGGLLKPIVSGTYAVNQKMLEDLKVGVQGQHASNLGGIIANEIAKEINVPAYIVDPV
VVDELDEVSRISGMADIPRKSIFHALNQKAVARRYAKEVGKKYEDLNLIVVHMGGGTSVGTHKDG
RVIEVNNTLDGEGPFSPERSGGVPIGDLVRLCFSNKYTYEEVMKKINGKGGVVSYLNTIDFKAVV
DKALEGDKKCALIYEAFTFQVAKEIGKCSTVLKGNVDAIILTGGIAYNEHVCNAIEDRVKFIAPV
VRYGGEDELLALAEGGLRVLRGEEKAKEYK

FIGURE 22

A.

ATGATTAAGAGTTTTAATGAAATTATCATGAAGGTAAAGAGCAAAGAAATGAAAAAAGTTGCTGT
TGCTGTAGCACAAGACGAGCCAGTACTTGAAGCAGTACGCGATGCTAAGAAAAATGGTATTGCAG
ATGCTATTCTTGTTGGCGACCATGACGAAATCGTGTCAATCGCGCTTAAAATAGGCATGGATGTA
AATGATTTTGAAATAGTAAACGAGCCTAACGTTAAGAAAGCTGCTTTAAAGGCAGTAGAGCTGGT
ATCAACTGGAAAAGCTGATATGGTAATGAAGGGACTTGTAAATACAGCAACTTTCTTACGCTCTG
TATTAAACAAAGAAGTTGGACTGAGAACAGGAAAAACTATGTCTCACGTTGCAGTATTTGAAACT
GAGAAATTTGATCGTCTGTTATTTTTAACAGATGTTGCTTTCAATACTTATCCTGAATTAAAGGA
AAAAATTGATATCGTAAACAATTCAGTTAAGGTTGCACATGCAATAGGTATTGAAAATCCAAAGG
TTGCTCCAATTTGTGCAGTTGAGGTTATAAACCCTAAAATGCCATCAACACTTGATGCAGCAATG
CTTTCAAAAATGAGTGACAGAGGACAAATTAAAGGTTGTGTAGTTGACGGACCGTTAGCACTTGA
TATCGCTTTATCAGAAGAAGCAGCACATCATAAGGGCGTAACAGGAGAAGTTGCTGGAAAAGCTG
ATATCTTCTTAATGCCAAACATTGAAACAGGAAATGTAATGTATAAGACTTTAACATATACAACT
GATAGCAAAAATGGCGGAATCTTAGTTGGAACTTCTGCACCAGTTGTTTTAACTTCACGCGCTGA
CAGCCATGAAACAAAAATGAACTCTATTGCACTTGCAGCTTTAGTTGCAGGCAATAAATAA

B.

ATGATTAAGAGTTTTAATGAAATTATCATGAAGGTAAAGAGCAAAGAAATGAAAAAAGTTGCTGT
TGCTGTAGCACAAGACGAGCCAGTACTTGAAGCAGTACGCGATGCTAAGAAAAATGGTATTGCCG
ATGCTATTCTGGTTGGCGACCATGACGAAATCGTGTCTATCGCGCTGAAAATAGGCATGGATGTA
AATGATTTTGAAATTGTTAACGAGCCTAACGTTAAGAAAGCTGCGTTAAAGGCAGTAGAGCTGGT
ATCAACTGGAAAAGCTGATATGGTAATGAAGGGACTGGTAAATACCGCAACTTTCTTACGCTCTG
TATTAAACAAAGAAGTTGGTCTGCGTACAGGAAAAACCATGTCTCACGTTGCAGTATTTGAAACT
GAGAAATTTGATCGTCTGTTATTTTTAACAGATGTTGCTTTCAATACTTATCCTGAATTAAAGGA
AAAAATTGATATCGTTAACAATAGCGTTAAGGTTGCACATGCCATTGGTATTGAAAATCCAAAGG
TTGCTCCAATTTGTGCAGTTGAGGTTATTAACCCGAAAATGCCATCAACACTTGATGCAGCAATG
CTTTCAAAAATGAGTGACCGCGGACAAATTAAAGGTTGTGTAGTTGACGGACCGCTGGCACTTGA
TATCGCTTTATCAGAAGAAGCAGCACATCATAAAGGCGTAACAGGAGAAGTTGCTGGAAAAGCTG
ATATCTTCTTAATGCCAAACATTGAAACAGGAAATGTAATGTATAAGACGTTAACCTATACCACT
GATAGCAAAAATGGCGGCATCCTGGTTGGAACTTCTGCACCAGTTGTTTTAACTTCACGCGCTGA
CAGCCATGAAACAAAAATGAACTCTATTGCACTGGCAGCGCTGGTTGCAGGCAATAAATAA

FIGURE 23

C.

ATGATTAAGAGTTTTAATGAAATTATCATGAAGGTAAAGAGCAAAGAAATGAAAAAAGTTGCTGT
TGCTGTTGCACAAGACGAGCCGGTACTGGAAGCGGTACGCGATGCTAAGAAAAATGGTATTGCCG
ATGCTATTCTGGTTGGCGACCATGACGAAATCGTCTCTATCGCGCTGAAAATTGGCATGGATGTT
AATGATTTTGAAATTGTTAACGAGCCTAACGTTAAGAAAGCTGCGCTGAAGGCGGTAGAGCTGGT
TTCCACCGGAAAAGCTGATATGGTAATGAAAGGGCTGGTGAATACCGCAACTTTCTTACGCAGCG
TACTGAACAAAGAAGTTGGTCTGCGTACCGGAAAAACCATGAGTCACGTTGCGGTATTTGAAACT
GAGAAATTTGATCGTCTGCTGTTTCTGACCGATGTTGCTTTCAATACTTATCCTGAATTAAAAGA
AAAAATTGATATCGTTAACAATAGCGTTAAGGTTGCGCATGCCATTGGTATTGAAAATCCAAAGG
TTGCTCCAATTTGTGCAGTTGAGGTTATTAACCCGAAAATGCCATCAACACTTGATGCCGCAATG
CTTAGCAAAATGAGTGACCGCGGACAAATTAAAGGTTGTGTGGTTGACGGCCCGCTGGCACTGGA
TATCGCGTTAAGCGAAGAAGCGGCACATCATAAAGGCGTAACCGGCGAAGTTGCTGGAAAAGCTG
ATATCTTCCTGATGCCAAACATTGAAACAGGCAATGTAATGTATAAAACGTTAACCTATACCACT
GATAGCAAAAATGGCGGCATCCTGGTTGGAACTTCTGCACCAGTTGTTTTAACCTCACGCGCTGA
CAGCCATGAAACCAAAATGAACAGCATTGCACTGGCAGCGCTGGTTGCAGGCAATAAATAA

D.

ATGATTAAAAGTTTTAACGAAATTATCATGAAAGTGAAAAGCAAAGAGATGAAAAAAGTGGCGGT
TGCGGTTGCGCAGGATGAACCGGTGCTGGAAGCGGTGCGCGATGCCAAAAAAAACGGTATTGCCG
ATGCCATTCTGGTGGGCGATCACGATGAAATTGTCTCTATTGCGCTGAAAATTGGCATGGATGTT
AACGATTTTGAAATTGTTAATGAACCGAACGTGAAAAAAGCGGCGCTGAAAGCGGTTGAACTGGT
TTCCACCGGTAAAGCCGATATGGTGATGAAAGGGCTGGTGAATACCGCAACCTTCCTGCGCAGCG
TGCTGAATAAAGAAGTGGGTCTGCGTACCGGTAAAACCATGAGTCATGTTGCGGTGTTTGAAACC
GAAAAATTTGACCGTCTGCTGTTTCTGACCGATGTTGCGTTTAATACCTATCCGGAACTGAAAGA
GAAAATTGATATCGTTAATAACAGCGTGAAAGTGGCGCATGCCATTGGTATTGAAAACCCGAAAG
TGGCGCCGATTTGCGCGGTTGAAGTGATTAACCCGAAAATGCCGTCAACGCTGGATGCCGCGATG
CTCAGCAAAATGAGCGATCGCGGTCAAATCAAAGGCTGTGTGGTTGATGGCCCGCTGGCGCTGGA
TATCGCGCTTAGCGAAGAAGCGGCGCATCATAAAGGCGTGACCGGCGAAGTGGCCGGTAAAGCCG
ATATTTTCCTGATGCCGAATATTGAAACCGGCAACGTGATGTATAAAACGCTGACCTATACCACC
GACAGCAAAAACGGCGGCATTCTGGTGGGTACCAGCGCGCCGGTGGTGCTGACCTCGCGCGCCGA
CAGCCATGAAACCAAAATGAACAGCATTGCGCTGGCGGCGCTGGTGGCCGGTAATAAATAA

FIGURE 23 (cont'd)

A.

ATGTATCGTTTACTGATTATCAATCCTGGCTCGACCTCAACTAAAATTGGTATTTATGACGATGA
AAAAGAGATATTTGAGAAGACTTTACGTCATTCAGCTGAAGAGATAGAAAAATATAACACTATAT
TTGATCAATTTCAGTTCAGAAAGAATGTAATTCTCGATGCGTTAAAAGAAGCAAACATTGAAGTA
AGTTCTTTAAATGCTGTAGTTGGACGCGGCGGACTGTTAAAGCCAATAGTAAGTGGAACTTATGC
AGTAAATCAAAAAATGCTTGAAGACCTTAAAGTAGGCGTTCAAGGTCAGCATGCGTCAAATCTTG
GTGGAATTATTGCAAATGAAATAGCAAAAGAAATAAATGTTCCAGCATACATCGTTGATCCAGTT
GTTGTGGATGAGCTTGATGAAGTTTCACGTATATCAGGAATGGCTGACATTCCACGTAAAAGTAT
ATTCCATGCATTAAATCAAAAAGCAGTTGCTAGACGCTATGCAAAAGAAGTTGGAAAAAAATACG
AAGATCTTAATTTAATCGTGGTCCACATGGGTGGCGGTACTTCAGTAGGTACTCATAAAGATGGT
AGAGTAATTGAAGTTAATAATACACTTGATGGAGAAGGTCCATTCTCACCAGAAAGAAGTGGTGG
CGTTCCAATAGGCGATCTTGTACGTTTGTGCTTCAGCAACAAATATACTTATGAAGAAGTAATGA
AAAAGATAAACGGCAAAGGCGGCGTTGTTAGTTACTTAAATACTATCGATTTTAAGGCTGTAGTT
GATAAAGCTCTTGAAGGCGATAAGAAATGTGCACTTATATATGAAGCTTTCACATTCCAGGTAGC
AAAAGAGATAGGAAAATGTTCAACCGTTTTAAAAGGAAATGTAGATGCAATAATCTTAACAGGCG
GAATTGCGTACAACGAGCATGTATGTAATGCCATAGAGGATAGAGTAAAATTCATTGCACCTGTA
GTTCGTTATGGTGGAGAAGATGAACTTCTTGCACTTGCAGAAGGTGGACTGCGCGTTTTACGCGG
AGAAGAAAAAGCTAAGGAATACAAATAA

B.

ATGTATCGTTTACTGATTATCAATCCTGGCTCGACCTCAACTAAAATTGGTATTTATGACGATGA
AAAAGAGATATTTGAGAAGACGTTACGTCATTCAGCTGAAGAGATTGAAAAATATAACACTATAT
TTGATCAATTTCAGTTCCGCAAGAATGTGATTCTCGATGCGTTAAAAGAAGCAAACATTGAAGTC
AGTTCTTTAAATGCTGTAGTTGGACGCGGCGGACTGTTAAAGCCAATTGTCAGTGGAACTTATGC
AGTAAATCAAAAAATGCTTGAAGACCTTAAAGTGGGCGTTCAAGGTCAGCATGCCAGCAATCTTG
GTGGCATTATTGCCAATGAAATCGCAAAAGAAATCAATGTTCCAGCATACATCGTTGATCCGGTT
GTTGTGGATGAGCTTGATGAAGTTAGCCGTATAAGCGGAATGGCTGACATTCCACGTAAAAGTAT
ATTCCATGCATTAAATCAAAAAGCAGTTGCTCGTCGCTATGCAAAAGAAGTTGGTAAAAAATACG
AAGATCTTAATTTAATCGTGGTCCACATGGGTGGCGGTACTTCAGTAGGTACTCATAAAGATGGT
CGCGTGATTGAAGTTAATAATACACTTGATGGCGAAGGTCCATTCTCACCAGAACGTAGTGGTGG
CGTTCCAATTGGCGATCTGGTACGTTTGTGCTTCAGCAACAAATATACTTATGAAGAAGTGATGA
AAAAGATAAACGGCAAAGGCGGCGTTGTTAGTTACCTGAATACTATCGATTTTAAGGCTGTAGTT
GATAAAGCGCTTGAAGGCGATAAGAAATGTGCACTGATTTATGAAGCTTTCACCTTCCAGGTAGC
AAAAGAGATTGGTAAATGTTCAACCGTTTTAAAAGGAAATGTTGATGCCATTATCTTAACAGGCG
GCATTGCTTACAACGAGCATGTATGTAATGCCATTGAGGATCGCGTAAAATTCATTGCACCTGTA
GTTCGTTATGGTGGCGAAGATGAACTGCTGGCACTGGCAGAAGGTGGACTGCGCGTTTTACGCGG
CGAAGAAAAAGCGAAGGAATACAAATAA

FIGURE 24

C.

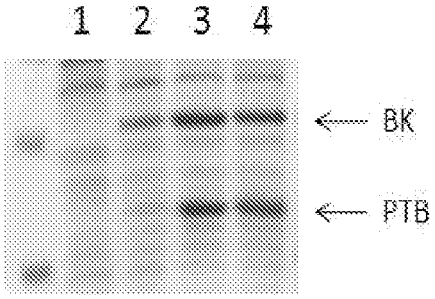
ATGTATCGTCTGCTGATTATCAATCCTGGCTCGACCTCAACTAAAATTGGTATTTATGACGATGA
AAAAGAGATATTTGAGAAAACGTTACGTCATAGCGCTGAAGAGATTGAAAAATATAACACTATTT
TTGATCAATTTCAGTTCCGCAAGAATGTGATTCTCGATGCGCTGAAAGAAGCAAACATTGAAGTC
AGTTCGCTGAATGCGGTAGTTGGTCGCGGCGGTCTGCTGAAGCCAATTGTCAGCGGCACTTATGC
GGTAAATCAAAAAATGCTGGAAGACCTGAAAGTGGGCGTTCAGGGGCAGCATGCCAGCAATCTTG
GTGGCATTATTGCCAATGAAATCGCCAAAGAAATCAATGTTCCGGCATACATCGTTGATCCGGTT
GTTGTGGATGAGCTGGATGAAGTTAGCCGTATCAGCGGAATGGCTGACATTCCACGTAAAAGTAT
TTTCCATGCACTGAATCAAAAAGCGGTTGCGCGTCGCTATGCAAAAGAAGTTGGTAAAAAATACG
AAGATCTTAATCTGATCGTGGTGCATATGGGTGGCGGTACTAGCGTCGGTACTCATAAAGATGGT
CGCGTGATTGAAGTTAATAATACACTTGATGGCGAAGGTCCATTCTCACCAGAACGTAGCGGTGG
CGTTCCAATTGGCGATCTGGTACGTTTGTGCTTCAGCAACAAATATACCTATGAAGAAGTGATGA
AAAAGATAAACGGCAAAGGCGGCGTTGTTAGTTACCTGAATACTATCGATTTTAAGGCGGTAGTT
GATAAAGCGCTGGAAGGCGATAAGAAATGTGCACTGATTTATGAAGCGTTCACCTTCCAGGTGGC
AAAAGAGATTGGTAAATGTTCAACCGTTCTGAAAGGCAATGTTGATGCCATTATCCTGACCGGCG
GCATTGCTTACAACGAGCATGTTTGTAATGCCATTGAGGATCGCGTAAAATTCATTGCACCTGTG
GTTCGTTATGGTGGCGAAGATGAACTGCTGGCACTGGCAGAAGGTGGTCTGCGCGTTTTACGCGG
CGAAGAAAAAGCGAAAGAATACAAATAA

D.

ATGTATCGTCTGCTGATTATCAACCCGGGCAGCACCTCAACCAAAATTGGTATTTACGACGATGA
AAAAGAGATTTTTGAAAAAACGCTGCGTCACAGCGCAGAAGAGATTGAAAAATACAACACCATTT
TCGATCAGTTCCAGTTCCGCAAAAACGTGATTCTCGATGCGCTGAAAGAAGCCAATATTGAAGTC
TCCTCGCTGAATGCGGTGGTCGGTCGCGGCGGTCTGCTGAAACCGATTGTCAGCGGCACTTATGC
GGTTAATCAGAAAATGCTGGAAGATCTGAAAGTGGGCGTGCAGGGGCAGCATGCCAGCAATCTCG
GCGGCATTATCGCCAATGAAATCGCCAAAGAGATCAACGTGCCGGCTTATATCGTCGATCCGGTG
GTGGTTGATGAACTGGATGAAGTCAGCCGTATCAGCGGCATGGCGGATATTCCGCGTAAAAGCAT
TTTCCATGCGCTGAATCAGAAAGCGGTTGCGCGTCGCTATGCCAAAGAAGTGGGTAAAAAATATG
AAGATCTCAATCTGATTGTGGTGCATATGGGCGGCGGCACCAGCGTCGGTACGCATAAAGATGGT
CGCGTGATTGAAGTGAATAACACGCTGGATGGCGAAGGGCCGTTCTCGCCGGAACGTAGCGGCGG
CGTGCCGATTGGCGATCTGGTGCGTCTGTGTTTCAGCAATAAATACACCTACGAAGAAGTGATGA
AAAAAATCAACGGCAAAGGCGGCGTGGTTAGCTATCTGAATACCATCGATTTTAAAGCGGTGGTT
GATAAAGCGCTGGAAGGCGATAAAAAATGCGCGCTGATTTATGAAGCGTTTACCTTCCAGGTGGC
GAAAGAGATTGGTAAATGTTCAACCGTGCTGAAAGGCAACGTTGATGCCATTATTCTGACCGGCG
GCATTGCTTATAACGAACATGTTTGTAATGCCATTGAAGATCGCGTGAAATTTATTGCGCCGGTG
GTGCGTTACGGCGGCGAAGATGAACTGCTGGCGCTGGCGGAAGGCGGTCTGCGCGTGCTGCGCGG
CGAAGAAAAAGCGAAAGAGTACAAATAA

FIGURE 24 (cont'd)

A.



B.

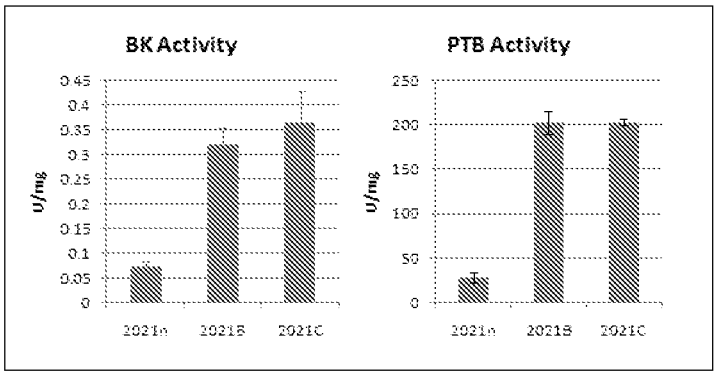


FIGURE 25

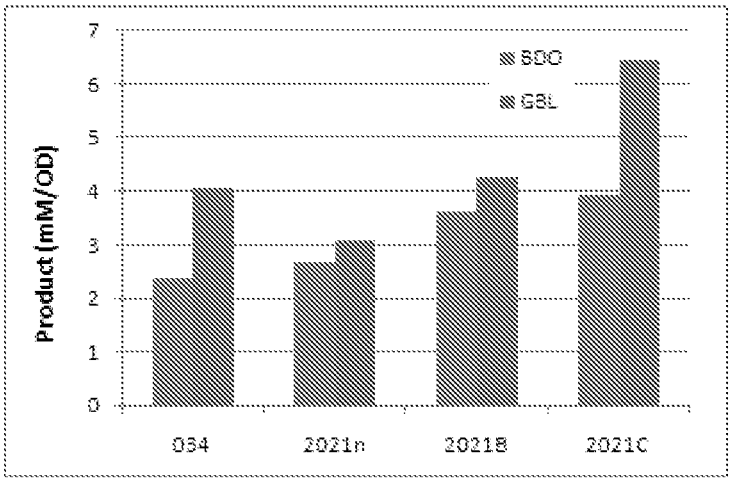


FIGURE 26

A.

ATGAATAAAGACACACTAATACCTACAACTAAAGATTTAAAAGTAAAAACAAATGGTGAAAACAT
TAATTTAAAGAACTACAAGGATAATTCTTCATGTTTCGGAGTATTCGAAAATGTTGAAAATGCTA
TAAGCAGCGCTGTACACGCACAAAAGATATTATCCCTTCATTATACAAAAGAGCAAAGAGAAAAA
ATCATAACTGAGATAAGAAAGGCCGCATTACAAAATAAAGAGGTCTTGGCTACAATGATTCTAGA
AGAAACACATATGGGAAGATATGAGGATAAAATATTAAAACATGAATTGGTAGCTAAATATACTC
CTGGTACAGAAGATTTAACTACTACTGCTTGGTCAGGTGATAATGGTCTTACAGTTGTAGAAATG
TCTCCATATGGTGTTATAGGTGCAATAACTCCTTCTACGAATCCAACTGAAACTGTAATATGTAA
TAGCATAGGCATGATAGCTGCTGGAAATGCTGTAGTATTTAACGGACACCCATGCGCTAAAAAAT
GTGTTGCCTTTGCTGTTGAAATGATAAATAAGGCAATTATTTCATGTGGCGGTCCTGAAAATCTA
GTAACAACTATAAAAAATCCAACTATGGAGTCTCTAGATGCAATTATTAAGCATCCTTCAATAAA
ACTTCTTTGCGGAACTGGGGGTCCAGGAATGGTAAAAACCCTCTTAAATTCTGGTAAGAAAGCTA
TAGGTGCTGGTGCTGGAAATCCACCAGTTATTGTAGATGATACTGCTGATATAGAAAAGGCTGGT
AGGAGCATCATTGAAGGCTGTTCTTTTGATAATAATTTACCTTGTATTGCAGAAAAAGAAGTATT
TGTTTTTGAGAATGTTGCAGATGATTTAATATCTAACATGCTAAAAAATAATGCTGTAATTATAA
ATGAAGATCAAGTATCAAAATTAATAGATTTAGTATTACAAAAAAATAATGAAACTCAAGAATAC
TTTATAAACAAAAAATGGGTAGGAAAAGATGCAAAATTATTCTTAGATGAAATAGATGTTGAGTC
TCCTTCAAATGTTAAATGCATAATCTGCGAAGTAAATGCAAATCATCCATTTGTTATGACAGAAC
TCATGATGCCAATATTGCCAATTGTAAGAGTTAAAGATATAGATGAAGCTATTAAATATGCAAAG
ATAGCAGAACAAAATAGAAAACATAGTGCCTATATTTATTCTAAAAATATAGACAACCTAAATAG
ATTTGAAAGAGAAATAGATACTACTATTTTTGTAAAGAATGCTAAATCTTTTGCTGGTGTTGGTT
ATGAAGCAGAAGGATTTACAACTTTCACTATTGCTGGATCTACTGGTGAGGGAATAACCTCTGCA
AGGAATTTTACAAGACAAAGAAGATGTGTACTTGCCGGCTAA

B.

MNKDTLIPTTKDLKVKTNGENINLKNYKDNSSCFGVFENVENAISSAVHAQKILSLHYTKEQREK
IITEIRKAALQNKEVLATMILEETHMGRYEDKILKHELVAKYTPGTEDLTTTAWSGDNGLTVVEM
SPYGVIGAITPSTNPTETVICNSIGMIAAGNAVVFNGHPCAKKCVAFAVEMINKAIISCGGPENL
VTTIKNPTMESLDAIIKHPSIKLLCGTGGPGMVKTLLNSGKKAIGAGAGNPPVIVDDTADIEKAG
RSIIEGCSFDNNLPCIAEKEVFVFENVADDLISNMLKNNAVIINEDQVSKLIDLVLQKNNETQEY
FINKKWVGKDAKLFLDEIDVESPSNVKCIICEVNANHPFVMTELMMPILPIVRVKDIDEAIKYAK
IAEQNRKHSAYIYSKNIDNLNRFEREIDTTIFVKNAKSFAGVGYEAEGFTTFTIAGSTGEGITSA
RNFTRQRRCVLAG

FIGURE 27

A.

ATGAATAAAGACACACTAATACCTACAACTAAAGATTTAAAAGTAAAAACAAATGGTGAAAACAT
TAATTTAAAGAACTACAAGGATAATTCTTCATGTTTCGGCGTATTCGAAAATGTTGAAAATGCTA
TAAGCAGCGCTGTACACGCACAAAAGATATTATCCCTTCATTATACAAAAGAGCAACGTGAAAAA
ATCATAACTGAGATAAGAAAGGCCGCATTACAAAATAAAGAGGTCTTGGCTACAATGATTCTGGA
AGAAACACATATGGGACGTTATGAGGATAAAATATTAAAACATGAATTGGTAGCTAAATATACTC
CTGGTACAGAAGATTTAACTACTACTGCCTGGTCAGGTGATAATGGTCTGACAGTTGTAGAAATG
TCTCCATATGGTGTTATTGGTGCAATAACTCCTTCTACGAATCCAACTGAAACTGTAATATGTAA
TAGCATAGGCATGATTGCTGCTGGAAATGCTGTAGTATTTAACGGACACCCATGCGCTAAAAAAT
GTGTTGCCTTTGCTGTTGAAATGATAAATAAGGCAATTATTTCATGTGGCGGTCCTGAAAATCTG
GTAACAACTATAAAAAATCCAACCATGGAGTCTCTGGATGCAATTATTAAGCATCCTTCAATAAA
ACTTCTTTGCGGAACTGGGGGTCCAGGAATGGTAAAAACCCTGTTAAATTCTGGTAAGAAAGCTA
TAGGTGCTGGTGCTGGAAATCCACCAGTTATTGTCGATGATACTGCTGATATAGAAAAGGCTGGT
CGTAGCATCATTGAAGGCTGTTCTTTTGATAATAATTTACCTTGTATTGCAGAAAAAGAAGTATT
TGTTTTTGAGAATGTTGCAGATGATTTAATATCTAACATGCTAAAAAATAATGCTGTAATTATAA
ATGAAGATCAAGTATCAAAATTAATCGATTTAGTATTACAAAAAAATAATGAAACTCAAGAATAC
TTTATAAACAAAAAATGGGTAGGAAAAGATGCAAAATTATTCCTCGATGAAATAGATGTTGAGTC
TCCTTCAAATGTTAAATGCATAATCTGCGAAGTAAATGCAAATCATCCATTTGTTATGACAGAAC
TGATGATGCCAATATTGCCAATTGTACGCGTTAAAGATATCGATGAAGCTATTAAATATGCAAAG
ATAGCAGAACAAAATAGAAAACATAGTGCCTATATTTATTCTAAAAATATCGACAACCTGAATCG
CTTTGAACGTGAAATAGATACTACTATTTTTGTAAAGAATGCTAAATCTTTTGCTGGTGTTGGTT
ATGAAGCAGAAGGATTTACAACTTTCACTATTGCTGGATCTACTGGTGAGGGAATAACCTCTGCA
CGTAATTTTACACGCCAACGTCGCTGTGTACTTGCCGGCTAA

B.

ATGAATAAAGACACACTGATCCCTACAACTAAAGATTTAAAAGTAAAAACAAATGGTGAAAACAT
TAATTTAAAGAACTACAAAGATAATAGCAGTTGTTTCGGCGTATTCGAAAATGTTGAAAATGCTA
TCAGCAGCGCTGTACACGCACAAAAGATATTATCGCTGCATTATACAAAAGAGCAACGTGAAAAA
ATCATCACTGAGATACGTAAGGCCGCATTACAAAATAAAGAGGTGCTGGCTACAATGATTCTGGA
AGAAACACATATGGGACGTTATGAGGATAAAATATTAAAACATGAACTGGTAGCTAAATATACTC
CTGGTACAGAAGATTTAACTACTACTGCCTGGAGCGGTGATAATGGTCTGACAGTTGTAGAAATG
TCTCCATATGGTGTTATTGGTGCAATAACTCCTTCTACCAATCCAACTGAAACTGTAATTTGTAA
TAGCATTGGCATGATTGCTGCTGGAAATGCTGTAGTATTTAACGGACACCCATGCGCTAAAAAAT
GTGTTGCCTTTGCTGTTGAAATGATCAATAAGGCAATTATTAGCTGTGGCGGTCCGGAAAATCTG
GTAACAACTATAAAAAATCCAACCATGGAGTCTCTGGATGCCATTATTAAGCATCCTTCAATAAA
ACTGCTTTGCGGAACTGGCGGTCCAGGAATGGTAAAAACCCTGTTAAATTCTGGTAAGAAAGCTA
TTGGTGCTGGTGCTGGAAATCCACCAGTTATTGTCGATGATACTGCTGATATTGAAAAGGCTGGT
CGTAGCATCATTGAAGGCTGTTCTTTTGATAATAATTTACCTTGTATTGCAGAAAAAGAAGTATT
TGTTTTTGAGAATGTTGCAGATGATTTAATATCTAACATGCTGAAAAATAATGCTGTAATTATCA
ATGAAGATCAGGTATCAAAATTAATCGATTTAGTATTACAAAAAAATAATGAAACTCAAGAATAC
TTTATCAACAAAAAATGGGTAGGTAAAGATGCAAAATTATTCCTCGATGAAATCGATGTTGAGTC
TCCTTCAAATGTTAAATGCATTATCTGCGAAGTGAATGCCAATCATCC

FIGURE 28

ATTTGTTATGACAGAACTGATGATGCCAATATTGCCAATTGTGCGCGTTAAAGATATCGATGAAG
CTATTAAATATGCAAAGATTGCAGAACAAAATAGAAAACATAGTGCCTATATTTATAGCAAAAAT
ATCGACAACCTGAATCGCTTTGAACGTGAAATCGATACTACTATTTTTGTAAAGAATGCTAAATC
TTTTGCTGGTGTTGGTTATGAAGCAGAAGGATTTACCACTTTCACTATTGCTGGATCTACTGGTG
AGGGCATAACCTCTGCACGTAATTTTACCCGCCAACGTCGCTGTGTACTGGCCGGCTAA

C.

ATGAATAAAGACACGCTGATCCCGACAACTAAAGATCTGAAAGTAAAAACCAATGGTGAAAACAT
TAATCTGAAGAACTACAAAGATAATAGCAGTTGTTTCGGCGTATTCGAAAATGTTGAAAATGCTA
TCAGCAGCGCGGTACACGCACAAAAGATACTCTCGCTGCATTATACCAAAGAGCAACGTGAAAAA
ATCATCACTGAGATCCGTAAGGCCGCATTACAAAATAAAGAGGTGCTGGCAACAATGATTCTGGA
AGAAACACATATGGGACGTTATGAGGATAAAATACTGAAACATGAACTGGTGGCGAAATATACGC
CTGGTACTGAAGATTTAACCACCACTGCCTGGAGCGGTGATAATGGTCTGACCGTTGTGGAAATG
TCGCCTTATGGTGTTATTGGTGCAATTACGCCTTCAACCAATCCAACTGAAACGGTAATTTGTAA
TAGCATTGGCATGATTGCTGCTGGAAATGCGGTAGTATTTAACGGTCACCCCTGCGCTAAAAAAT
GTGTTGCCTTTGCTGTTGAAATGATCAATAAAGCGATTATTAGCTGTGGCGGTCCGGAAAATCTG
GTAACCACTATAAAAAATCCAACCATGGAGTCGCTGGATGCCATTATTAAGCATCCTTCAATCAA
ACTGCTGTGCGGCACTGGCGGTCCAGGAATGGTGAAAACCCTGCTGAATAGCGGTAAGAAAGCGA
TTGGTGCTGGTGCTGGAAATCCACCAGTTATTGTCGATGATACTGCTGATATTGAAAAAGCGGGT
CGTAGCATCATTGAAGGCTGTTCTTTTGATAATAATTTACCTTGTATTGCAGAAAAAGAAGTATT
TGTTTTTGAGAATGTTGCCGATGATCTGATCTCTAACATGCTGAAAAATAATGCGGTGATTATCA
ATGAAGATCAGGTTAGCAAACTGATCGATCTGGTATTACAAAAAAATAATGAAACTCAAGAATAC
TTTATCAACAAAAAATGGGTAGGTAAAGATGCAAAACTGTTCCTCGATGAAATCGATGTTGAGTC
GCCTTCAAATGTTAAATGCATTATCTGCGAAGTGAATGCCAATCATCCATTTGTGATGACCGAAC
TGATGATGCCAATTTTGCCGATTGTGCGCGTTAAAGATATCGATGAAGCGATTAAATATGCAAAG
ATTGCAGAACAAAATCGTAAACATAGTGCCTATATTTATAGCAAAAATATCGACAACCTGAATCG
CTTTGAACGTGAAATCGATACCACTATTTTTGTGAAGAATGCTAAATCTTTTGCTGGTGTTGGTT
ATGAAGCAGAAGGTTTTACCACTTTCACTATTGCTGGAAGCACCGGTGAAGGCATTACCTCTGCA
CGTAATTTTACCCGCCAACGTCGCTGTGTACTGGCCGGCTAA

D.

ATGAATAAAGATACGCTGATCCCGACCACCAAAGATCTGAAAGTGAAAACCAACGGCGAAAATAT
CAACCTGAAAAACTATAAAGATAACAGCAGTTGCTTTGGCGTGTTTGAAAACGTTGAAAACGCCA
TCTCCAGCGCGGTGCATGCGCAAAAAATTCTCTCGCTGCATTACACCAAAGAGCAGCGTGAAAAA
ATTATCACCGAAATCCGTAAAGCGGCGCTGCAAAACAAAGAAGTGCTGGCAACCATGATCCTGGA
AGAAACGCATATGGGGCGTTATGAAGATAAAATTCTGAAACATGAACTGGTGGCGAAATACACGC
CGGGCACTGAAGATCTGACCACCACCGCCTGGAGCGGCGATAACGGCCTGACCGTGGTGGAGATG
TCGCCTTATGGCGTGATTGGCGCGATTACGCCGTCAACCAACCCGACCGAAACGGTGATTTGTAA
CAGCATTGGCATGATTGCCGCGGGTAATGCGGTGGTGTTTAACGGTCATCCCTGCGCGAAAAAAT
GTGTGGCGTTTGCCGTTGAGATGATCAACAAAGCGATTATCAGCTGCGGCGGCCCGGAAAATCTG
GTGACCACCATCAAAAATCCGACCATGGAATCGCTGGATGCCATTATCAAACATCCTTCCATCAA
ACTGCTGTGCGGCACCGGCGGCCCGGGCATGGTGAAAACGCTGCTGAACAGCGGTAAAAAAGCGA
TTGGCGCGGGCGCGGGTAACCCGCCGGTGATTGTCGATGACACCGCCGATATT

FIGURE 28 (cont'd)

GAAAAAGCGGGGCGTAGCATTATTGAAGGCTGTTCTTTTGATAACAACCTGCCCTGCATTGCCGA
AAAAGAAGTGTTTGTCTTTGAAAACGTCGCCGATGATCTGATCAGCAATATGCTGAAAAACAACG
CGGTGATTATCAATGAAGATCAGGTTAGCAAACTGATCGATCTGGTGCTGCAAAAAAACAACGAA
ACGCAGGAATATTTTATCAACAAAAAATGGGTTGGTAAAGATGCCAAACTGTTTCTCGATGAAAT
CGATGTTGAATCGCCGTCTAACGTGAAATGTATTATCTGCGAAGTGAACGCCAACCATCCGTTTG
TGATGACCGAACTGATGATGCCGATTCTGCCGATTGTGCGCGTGAAAGATATCGATGAAGCGATT
AAATATGCCAAAATTGCCGAACAAAACCGTAAACACAGCGCCTATATTTACAGCAAAAATATCGA
TAACCTGAACCGCTTTGAACGTGAAATCGATACCACCATTTTTGTGAAAAATGCCAAAAGTTTTG
CCGGCGTTGGTTATGAAGCGGAAGGTTTTACCACCTTTACCATTGCCGGTAGCACCGGCGAAGGC
ATTACCAGCGCCCGTAATTTTACCCGCCAGCGTCGCTGCGTGCTGGCGGGCTAA

FIGURE 28 (cont'd)

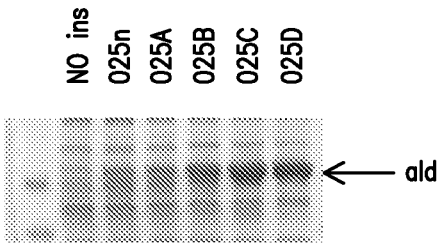


FIG. 29

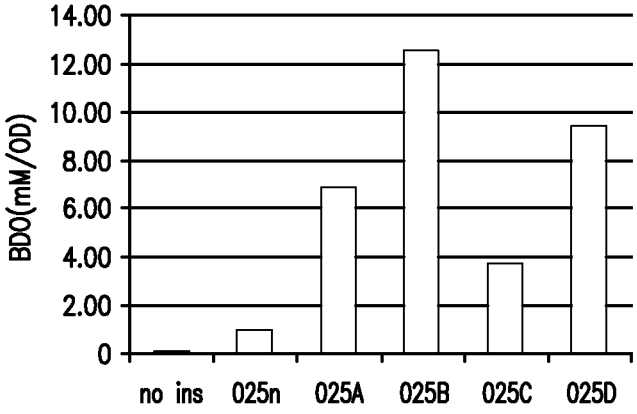


FIG. 30A

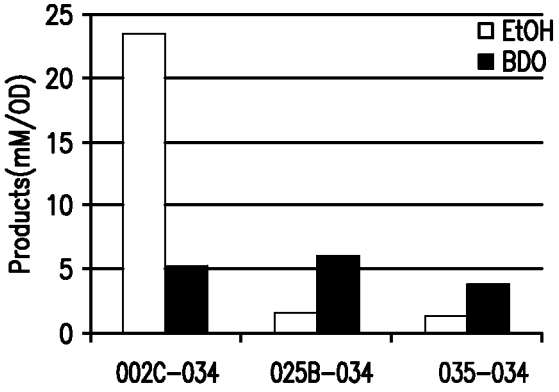


FIG. 30B

A.

ATGAAAGCTGCAGTAGTAGAGCAATTTAAGGAACCATTAAAAATTAAAGAAGTGGAAAAGCCATC
TATTTCATATGGCGAAGTATTAGTCCGCATTAAAGCATGCGGTGTATGCCATACGGACTTGCACG
CCGCTCATGGCGATTGGCCAGTAAAACCAAAACTTCCTTTAATCCCTGGCCATGAAGGAGTCGGA
ATTGTTGAAGAAGTCGGTCCGGGGGTAACCCATTTAAAAGTGGGAGACCGCGTTGGAATTCCTTG
GTTATATTCTGCGTGCGGCCATTGCGAATATTGTTTAAGCGGACAAGAAGCATTATGTGAACATC
AACAAAACGCCGGCTACTCAGTCGACGGGGGTTATGCAGAATATTGCAGAGCTGCGCCAGATTAT
GTGGTGAAAATTCCTGACAACTTATCGTTTGAAGAAGCTGCTCCTATTTTCTGCGCCGGAGTTAC
TACTTATAAAGCGTTAAAAGTCACAGGTACAAAACCGGGAGAATGGGTAGCGATCTATGGCATCG
GCGGCCTTGGACATGTTGCCGTCCAGTATGCGAAAGCGATGGGGCTTCATGTTGTTGCAGTGGAT
ATCGGCGATGAGAAACTGGAACTTGCAAAAGAGCTTGGCGCCGATCTTGTTGTAAATCCTGCAAA
AGAAAATGCGGCCCAATTTATGAAAGAGAAAGTCGGCGGAGTACACGCGGCTGTTGTGACAGCTG
TATCTAAACCTGCTTTTCAATCTGCGTACAATTCTATCCGCAGAGGCGGCACGTGCGTGCTTGTC
GGATTACCGCCGGAAGAAATGCCTATTCCAATCTTTGATACGGTATTAAACGGAATTAAAATTAT
CGGTTCCATTGTCGGCACGCGGAAAGACTTGCAAGAAGCGCTTCAGTTCGCTGCAGAAGGTAAAG
TAAAAACCATTATTGAAGTGCAACCTCTTGAAAAAATTAACGAAGTATTTGACAGAATGCTAAAA
GGAGAAATTAACGGACGGGTTGTTTTAACGTTAGAAAATAATAATTAA

B.

MKAAVVEQFKEPLKIKEVEKPSISYGEVLVRIKACGVCHTDLHAAHGDWPVKPKLPLIPGHEGVG
IVEEVGPGVTHLKVGDRVGIPWLYSACGHCEYCLSGQEALCEHQQNAGYSVDGGYAEYCRAAPDY
VVKIPDNLSFEEAAPIFCAGVTTYKALKVTGTKPGEWVAIYGIGGLGHVAVQYAKAMGLHVVAVD
IGDEKLELAKELGADLVVNPAKENAAQFMKEKVGGVHAAVVTAVSKPAFQSAYNSIRRGGTCVLV
GLPPEEMPIPIFDTVLNGIKIIGSIVGTRKDLQEALQFAAEGKVKTIIEVQPLEKINEVFDRMLK
GEINGRVVLTLENNN

FIGURE 31

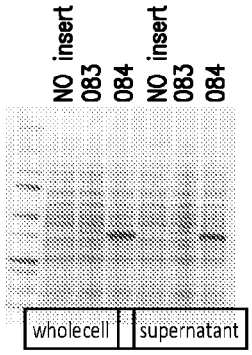


FIG. 32A

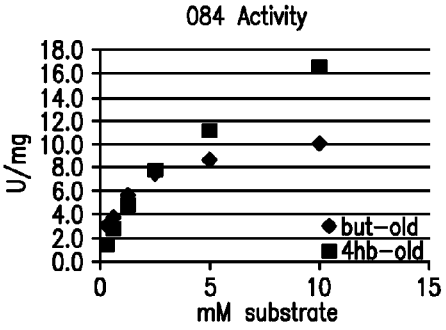


FIG. 32B

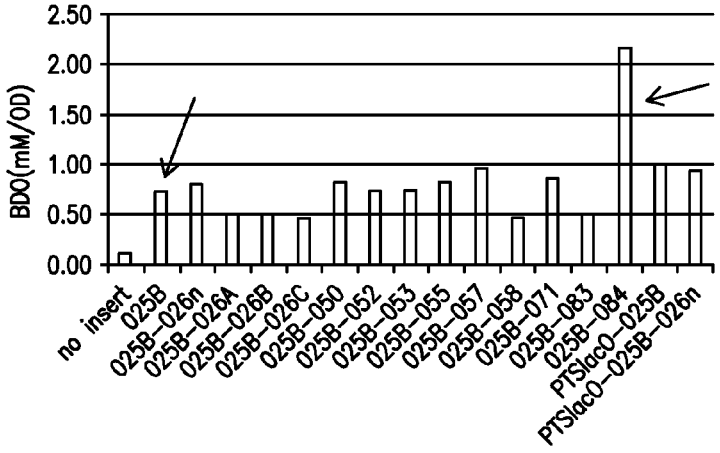


FIG. 33

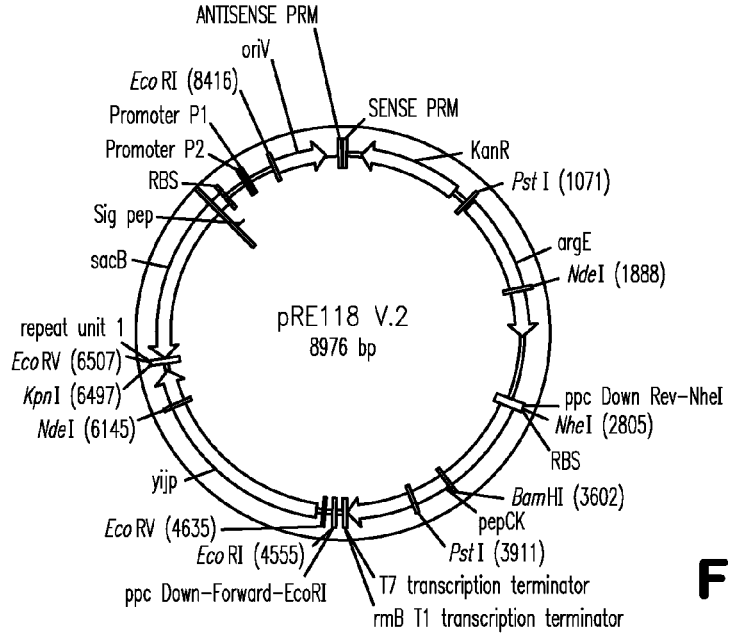


FIG. 34

A.

aTGGCTATCGAAATCAAAGTACCGGACATCGGGGCTGATGAAGTTGAAATCACCGAGATCCTGGTCAAA
GTGGGCGACAAAGTTGAAGCCGAACAGTCGCTGATCACCGTAGAAGGCGACAAAGCCTCTATGGAAGT
TCCGTCTCCGCAGGCGGGTATCGTTAAAGAGATCAAAGTCTCTGTTGGCGATAAAACCCAGACCGGCGC
ACTGATTATGATTTTCGATTCCGCCGACGGTGCAGCAGACGCTGCACCTGCTCAGGCAGAAGAGAAGAA
AGAAGCAGCTCCGGCAGCAGCACCAGCGGCTGCGGCGGCAAAAGACGTTAACGTTCCGGATATCGGCA
GCGACGAAGTTGAAGTGACCGAAATCCTGGTGAAAGTTGGCGATAAAGTTGAAGCTGAACAGTCGCTG
ATCACCGTAGAAGGCGACAAGGCTTCTATGGAAGTTCCGGCTCCGTTTGCTGGCACCGTGAAAGAGATC
AAAGTGAACGTGGGTGACAAAGTGTCTACCGGCTCGCTGATTATGGTCTTCGAAGTCGCGGGTGAAGC
AGGCGCGGCAGCTCCGGCCGCTAAACAGGAAGCAGCTCCGGCAGCGGCCCCTGCACCAGCGGCTGGC
GTGAAAGAAGTTAACGTTCCGGATATCGGCGGTGACGAAGTTGAAGTGACTGAAGTGATGGTGAAAGT
GGGCGACAAAGTTGCCGCTGAACAGTCACTGATCACCGTAGAAGGCGACAAAGCTTCTATGGAAGTTCC
GGCGCCGTTTGCAGGCGTCGTGAAGGAACTGAAAGTCAACGTTGGCGATAAAGTGAAAACTGGCTCGC
TGATTATGATCTTCGAAGTTGAAGGCGCAGCGCCTGCGGCAGCTCCTGCGAAACAGGAAGCGGCAGCG
CCGGCACCGGCAGCAAAAGCTGAAGCCCCGGCAGCAGCACCAGCTGCGAAAGCGGAAGGCAAATCTG
AATTTGCTGAAAACGACGCTTATGTTCACGCGACTCCGCTGATCCGCCGTCTGGCACGCGAGTTTGGTGT
TAACCTTGCGAAAGTGAAGGGCACTGGCCGTAAAGGTCGTATCCTGCGCGAAGACGTTCAGGCTTACGT
GAAAGAAGCTATCAAACGTGCAGAAGCAGCTCCGGCAGCGACTGGCGGTGGTATCCCTGGCATGCTGC
CGTGGCCGAAGGTGGACTTCAGCAAGTTTGGTGAAATCGAAGAAGTGGAACTGGGCCGCATCCAGAAA
ATCTCTGGTGCGAACCTGAGCCGTAACTGGGTAATGATCCCGCATGTTACTCACTTCGACAAAACCGATA
TCACCGAGTTGGAAGCGTTCCGTAAACAGCAGAACGAAGAAGCGGCGAAACGTAAGCTGGATGTGAAG
ATCACCCCGGTTGTCTTCATCATGAAAGCCGTTGCTGCAGCTCTTGAGCAGATGCCTCGCTTCAATAGTTC
GCTGTCGGAAGACGGTCAGCGTCTGACCCTGAAGAAATACATCAACATCGGTGTGGCGGTGGATACCC
CGAACGGTCTGGTTGTTCCGGTATTCAAAGACGTCAACAAGAAAGGCATCATCGAGCTGTCTCGCGAGC
TGATGACTATTTCTAAGAAAGCGCGTGACGGTAAGCTGACTGCGGGCGAAATGCAGGGCGGTTGCTTC
ACCATCTCCAGCATCGGCGGCCTGGGTACTACCCACTTCGCGCCGATTGTGAACGCGCCGGAAGTGGCT
ATCCTCGGCGTTTCCAAGTCCGCGATGGAGCCGGTGTGGAATGGTAAAGAGTTCGTGCCGCGTCTGATG
CTGCCGATTTCTCTCTCCTTCGACCACCGCGTGATCGACGGTGCTGATGGTGCCCGTTTCATTACCATCAT
TAACAACACGCTGTCTGACATTCGCCGTCTGGTGATGTAAGTAAAAGAGCCGGCCCAACGGCCGGCTTT
TTTCTGGTAATCTCATGAATGTATTGAGGTTATTAGCGAATAGACAAATCGGTTGCCGTTTGTTGTTTAAA
AATTGTTAACAATTTTGTAAAATACCGACGGATAGAACGACCCGGTGGTGGTTAGGGTATTACTTCACAT
ACCCTATGGATTTCTGGGTGCAGCAAGGTAGCAAGCGCCAGAATCCCCAGGAGCTTACATAAGTAAGTG
ACTGGGGTGAGGGCGTGAAGCTAACGCCGCTGCGGCCTGAAAGACGACGGGTATGACCGCCGGAGAT
AAATATATAGAGGTCATGATGAGTACTGAAATCAAAACTCAGGTCGTGGTACTTGGGGCAGGCCCCGCA
GGTTACTCCGCTGCCTTCCGTTGCGCTGATTTAGGTCTGGAAACCGTAATCGTAGAACGTTACAACACCC
TTGGCGGTGTTTGTCTGAACGTGGGTTGTATCCCTTCTAAAGCGCTGCTGCACGTGGCAAAAGTTATCGA
AGAAGCGAAAGCGCTGGCCGAACACGGCATCGTTTTCGGCGAACCGAAAACTGACATTGACAAGATCC
GCACCTGGAAAGAAAAAGTCATCACTCAGCTGACCGGTGGTCTGGCTGGCATGGCCAAAGGTCGTAAA
GTGAAGGTGGTTAACGGTCTGGGTAAATTTACCGGCGCTAACACCCTGGAAGTGGAAGGCGAAAACGG
CAAAACCGTGATCAACTTCGACAACGCCATCATCGCGGCGGGTTCCCGTCCGATTCAGCTGCCGTTTATC
CCGCATGAAGATCCGCGCGTATGGGACTCCACCGACGCGCTGGAACTGAAATCTGTACCGAAACGCATG
CTGGTGATGGGCGGCGGTATCATCGGTCTGGAAATGGGTACCGTATACCATGCGCTGGGTTCAGAGATT
GACGTGGTGGAAATGTTCGACCAGGTTATCCCGGCTGCCGACAAAGACGTGGTGAAAGTCTTCACCAAA
CGCATCAGCAAGAAATTTAACCTGATGCTGGAAGCCAAAGTGACTGCCGTTGAAGCGAAAGAAGACGG

FIGURE 35

TATTTACGTTTCCATGGAAGGTAAAAAAGCACCGGCGGAAGCGCAGCGTTACGACGCAGTGCTGGTCG
CTATCGGCCGCGTACCGAATGGTAAAAACCTCGATGCAGGTAAAGCTGGCGTGGAAGTTGACGATCGC
GGCTTCATCCGCGTTGACAAACAAATGCGCACCAACGTGCCGCACATCTTTGCTATCGGCGATATCGTCG
GTCAGCCGATGCTGGCGCACAAAGGTGTCCATGAAGGCCACGTTGCCGCAGAAGTTATCTCCGGTCTGA
AACACTACTTCGATCCGAAAGTGATCCCATCCATCGCCTACACTAAACCAGAAGTGGCATGGGTCGGTCT
GACCGAGAAAGAAGCGAAAGAGAAAGGCATCAGCTACGAAACCGCCACCTTCCCGTGGGCTGCTTCCG
GCCGTGCTATCGCTTCTGACTGCGCAGATGGTATGACCAAACTGATCTTCGACAAAGAGACCCACCGTG
TTATCGGCGGCGCGATTGTCGGCACCAACGGCGGCGAGCTGCTGGGTGAGATCGGCCTGGCTATCGAG
ATGGGCTGTGACGCTGAAGACATCGCCCTGACCATCCACGCTCACCCGACTCTGCACGAGTCCGTTGGC
CTGGCGGCGGAAGTGTTCGAAGGCAGCATCACCGACCTGCCAAACGCCAAAGCGAAGAAAAAGTAACT
TTTTCTTTCAGGAAAAAAGCATAAGCGGCTCCGGGAGCCGCTTTTTTTATGCCTGATGTTTAGAACTATG
TCACTGTTCATAAACCGCTACACCTCATACATACTTTAAGGGCGAATTCTGCAGATATCCATCACACTGGC
GGCCGCTCGAGCATGCATCTAGCACATCCGGCAATTAAAAAAGCGGCTAACCACGCCGCTTTTTTTACGT
CTGCAATTTACCTTTCCAGTCTTCTTGCTCCACGTTCAGAGAGACGTTCGCATACTGCTGACCGTTGCTCG
TTATTCAGCCTGACAGTATGGTTACTGTCGTTTAGACGTTGTGGGCGGCTCTCCTGAACTTTCTCCCGAA
AAACCTGACGTTGTTCAGGTGATGCCGATTGAACACGCTGGCGGGCGTTATCACGTTGCTGTTGATTCA
GTGGGCGCTGCTGTACTTTTTCCTT

FIGURE 35 (cont'd)

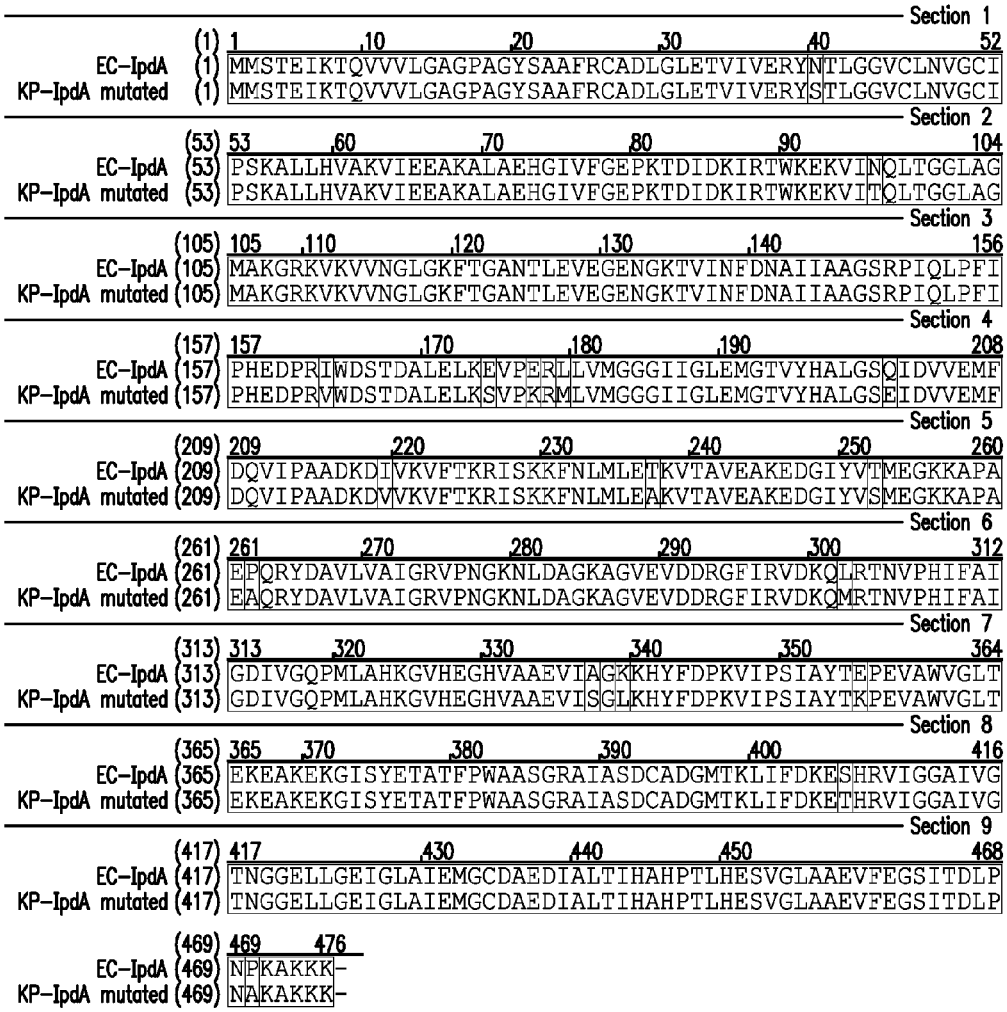

Section 1
EC-IpdA (1) MMSTEIKTQVVVLGAGPAGYSAAFRCADLGLETVIVERYNTLGGVCLNVGCI
KP-IpdA mutated (1) MMSTEIKTQVVVLGAGPAGYSAAFRCADLGLETVIVERYSTLGGVCLNVGCI
Section 2
EC-IpdA (53) PSKALLHVAKVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVINQLTGGLAG
KP-IpdA mutated (53) PSKALLHVAKVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVITQLTGGLAG
Section 3
EC-IpdA (105) MAKGRKVKVVNGLGKFTGANTLEVEGENGKTVINFDNAIIAAGSRPIQLPFI
KP-IpdA mutated (105) MAKGRKVKVVNGLGKFTGANTLEVEGENGKTVINFDNAIIAAGSRPIQLPFI
Section 4
EC-IpdA (157) PHEDPRIWDSTDALELKEVPERLLVMGGGIIGLEMGTVYHALGSQIDVVEMF
KP-IpdA mutated (157) PHEDPRVWDSTDALELKSVPKRMLVMGGGIIGLEMGTVYHALGSEIDVVEMF
Section 5
EC-IpdA (209) DQVIPAADKDIVKVFTKRISKKFNLMLETKVTAVEAKEDGIYVTMEGKKAPA
KP-IpdA mutated (209) DQVIPAADKDVVKVFTKRISKKFNLMLEAKVTAVEAKEDGIYVSMEGKKAPA
Section 6
EC-IpdA (261) EPQRYDAVLVAIGRVPNGKNLDAGKAGVEVDDRGFIRVDKQLRTNVPHIFAI
KP-IpdA mutated (261) EAQRYDAVLVAIGRVPNGKNLDAGKAGVEVDDRGFIRVDKQMRTNVPHIFAI
Section 7
EC-IpdA (313) GDIVGQPMLAHKGVHEGHVAAEVIAGKKHYFDPKVIPSIAYTEPEVAWVGLT
KP-IpdA mutated (313) GDIVGQPMLAHKGVHEGHVAAEVISGLKHYFDPKVIPSIAYTKPEVAWVGLT
Section 8
EC-IpdA (365) EKEAKEKGISYETATFPWAASGRAIASDCADGMTKLIFDKESHRVIGGAIVG
KP-IpdA mutated (365) EKEAKEKGISYETATFPWAASGRAIASDCADGMTKLIFDKETHRVIGGAIVG
Section 9
EC-IpdA (417) TNGGELLGEIGLAIEMGCDAEDIALTIHAHPTLHESVGLAAEVFEGSITDLP
KP-IpdA mutated (417) TNGGELLGEIGLAIEMGCDAEDIALTIHAHPTLHESVGLAAEVFEGSITDLP
EC-IpdA (469) NPKAKKK-
KP-IpdA mutated (469) NAKAKKK-

FIG. 36

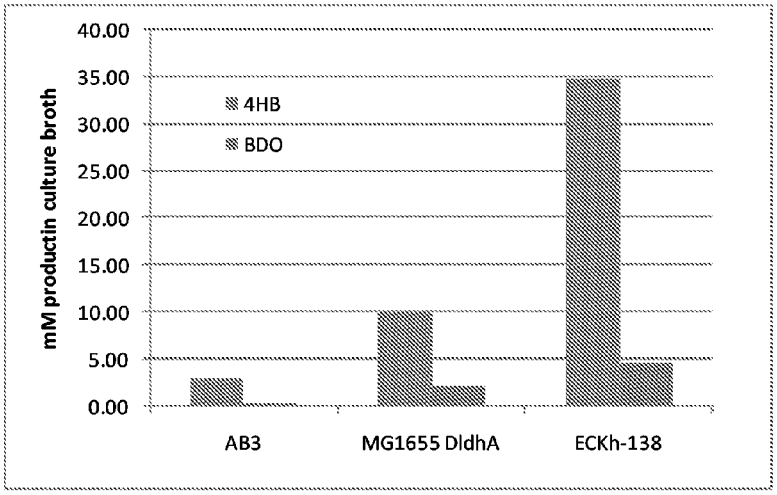


FIGURE 37

*atactatacatatgaaccat*gcgagttacgggcctat**aagccaggcgagat<u>atgatctatatcaa</u>tttctcatctataatgctttgtta**
gtatctcgtcgccgacttaataaagagagagttagtgtgaaagctgacaacccttttgatcttttacttcctgctgcaatggccaaagtgg
ccgaagaggcgggtgtctataaagcaacgaaacatccgcttaagactttctatctggcgattaccgccggtgttttcatctcaatcgcattc
accactggcacaggcacaGAAGGTAGGTGTTACatgtcagaacgtttacaca*atgacgtggatcctattattat*

FIGURE 38

AAGAGGTAAAAGAATAATGGCTATCGAAATCAAAGTACCGGACATCGGGGCTGATGAAGTTGAAATCA
CCGAGATCCTGGTCAAAGTGGGCGACAAAGTTGAAGCCGAACAGTCGCTGATCACCGTAGAAGGCGAC
AAAGCCTCTATGGAAGTTCCGTCTCCGCAGGCGGGTATCGTTAAAGAGATCAAAGTCTCTGTTGGCGAT
AAAACCCAGACCGGCGCACTGATTATGATTTTCGATTCCGCCGACGGTGCAGCAGACGCTGCACCTGCT
CAGGCAGAAGAGAAGAAAGAAGCAGCTCCGGCAGCAGCACCAGCGGCTGCGGCGGCAAAAGACGTTA
ACGTTCCGGATATCGGCAGCGACGAAGTTGAAGTGACCGAAATCCTGGTGAAAGTTGGCGATAAAGTT
GAAGCTGAACAGTCGCTGATCACCGTAGAAGGCGACAAGGCTTCTATGGAAGTTCCGGCTCCGTTTGCT
GGCACCGTGAAAGAGATCAAAGTGAACGTGGGTGACAAAGTGTCTACCGGCTCGCTGATTATGGTCTTC
GAAGTCGCGGGTGAAGCAGGCGCGGCAGCTCCGGCCGCTAAACAGGAAGCAGCTCCGGCAGCGGCCC
CTGCACCAGCGGCTGGCGTGAAAGAAGTTAACGTTCCGGATATCGGCGGTGACGAAGTTGAAGTGACT
GAAGTGATGGTGAAAGTGGGCGACAAAGTTGCCGCTGAACAGTCACTGATCACCGTAGAAGGCGACAA
AGCTTCTATGGAAGTTCCGGCGCCGTTTGCAGGCGTCGTGAAGGAACTGAAAGTCAACGTTGGCGATAA
AGTGAAAACTGGCTCGCTGATTATGATCTTCGAAGTTGAAGGCGCAGCGCCTGCGGCAGCTCCTGCGAA
ACAGGAAGCGGCAGCGCCGGCACCGGCAGCAAAAGCTGAAGCCCCGGCAGCAGCACCAGCTGCGAAA
GCGGAAGGCAAATCTGAATTTGCTGAAAACGACGCTTATGTTCACGCGACTCCGCTGATCCGCCGTCTG
GCACGCGAGTTTGGTGTTAACCTTGCGAAAGTGAAGGGCACTGGCCGTAAAGGTCGTATCCTGCGCGA
AGACGTTCAGGCTTACGTGAAAGAAGCTATCAAACGTGCAGAAGCAGCTCCGGCAGCGACTGGCGGTG
GTATCCCTGGCATGCTGCCGTGGCCGAAGGTGGACTTCAGCAAGTTTGGTGAAATCGAAGAAGTGGAA
CTGGGCCGCATCCAGAAAATCTCTGGTGCGAACCTGAGCCGTAACTGGGTAATGATCCCGCATGTTACT
CACTTCGACAAAACCGATATCACCGAGTTGGAAGCGTTCCGTAAACAGCAGAACGAAGAAGCGGCGAA
ACGTAAGCTGGATGTGAAGATCACCCCGGTTGTCTTCATCATGAAAGCCGTTGCTGCAGCTCTTGAGCA
GATGCCTCGCTTCAATAGTTCGCTGTCGGAAGACGGTCAGCGTCTGACCCTGAAGAAATACATCAACAT
CGGTGTGGCGGTGGATACCCCGAACGGTCTGGTTGTTCCGGTATTCAAAGACGTCAACAAGAAAGGCA
TCATCGAGCTGTCTCGCGAGCTGATGACTATTTCTAAGAAAGCGCGTGACGGTAAGCTGACTGCGGGCG
AAATGCAGGGCGGTTGCTTCACCATCTCCAGCATCGGCGGCCTGGGTACTACCCACTTCGCGCCGATTGT
GAACGCGCCGGAAGTGGCTATCCTCGGCGTTTCCAAGTCCGCGATGGAGCCGGTGTGGAATGGTAAAG
AGTTCGTGCCGCGTCTGATGCTGCCGATTTCTCTCTCCTTCGACCACCGCGTGATCGACGGTGCTGATGG
TGCCCGTTTCATTACCATCATTAACAACACGCTGTCTGACATTCGCCGTCTGGTGATGTAAGTAAAAGAG
CCGGCCCAACGGCCGGCTTTTTTCTGGTAATCTCATGAATGTATTGAGGTTATTAGCGAATAGACAAATC
GGTTGCCGTTTGTTAAGCCAGGCGAGATATGATCTATATCAATTTCTCATCTATAATGCTTTGTTAGTATC
TCGTCGCCGACTTAATAAAGAGAGAGTTAGTCTTCTATATCACAGCAAGAAGGTAGGTGTTACATGATG
AGTACTGAAATCAAAACTCAGGTCGTGGTACTTGGGGCAGGCCCCGCAGGTTACTCTGCAGCCTTCCGT
TGCGCTGATTTAGGTCTGGAAACCGTCATCGTAGAACGTTACAGCACCCTCGGTGGTGTTTGTCTGAACG
TGGGTTGTATCCCTTCTAAAGCGCTGCTGCACGTGGCAAAAGTTATCGAAGAAGCGAAAGCGCTGGCCG
AACACGGCATCGTTTTCGGCGAACCGAAAACTGACATTGACAAGATCCGCACCTGGAAAGAAAAAGTCA
TCACTCAGCTGACCGGTGGTCTGGCTGGCATGGCCAAAGGTCGTAAAGTGAAGGTGGTTAACGGTCTG
GGTAAATTTACCGGCGCTAACACCCTGGAAGTGGAAGGCGAAAACGGCAAAACCGTGATCAACTTCGA
CAACGCCATCATCGCGGCGGGTTCCCGTCCGATTCAGCTGCCGTTTATCCCGCATGAAGATCCGCGCGTA
TGGGACTCCACCGACGCGCTGGAACTGAAATCTGTACCGAAACGCATGCTGGTGATGGGCGGCGGTAT
CATCGGTCTGGAAATGGGTACCGTATACCATGCGCTGGGTTCAGAGATTGACGTGGTGGAAATGTTCGA
CCAGGTTATCCCGGCTGCCGACAAAGACGTGGTGAAAGTCTTCACCAAACGCATCAGCAAGAAATTTAA
CCTGATGCTGGAAGCCAAAGTGACTGCCGTTGAAGCGAAAGAAGACGGTATTTACGTTTCCATGGAAG
GTAAAAAAGCACCGGCGGAAGCGCAGCGTTACGACGCAGTGCTGGTCGCTATCGGCCGCGTACCGAAT
GGTAAAAACCTCGATGCAGGTAAAGCTGGCGTGGAAGTTGACGATCGCGGCTTCATCCGCGTTGACAA

FIGURE 39

ACAAATGCGCACCAACGTGCCGCACATCTTTGCTATCGGCGATATCGTCGGTCAGCCGATGCTGGCGCA
CAAAGGTGTCCATGAAGGCCACGTTGCCGCAGAAGTTATCTCCGGTCTGAAACACTACTTCGATCCGAA
AGTGATCCCATCCATCGCCTACACTAAACCAGAAGTGGCATGGGTCGGTCTGACCGAGAAAGAAGCGA
AAGAGAAAGGCATCAGCTACGAAACCGCCACCTTCCCGTGGGCTGCTTCCGGCCGTGCTATCGCTTCTG
ACTGCGCAGATGGTATGACCAAACTGATCTTCGACAAAGAGACCCACCGTGTTATCGGCGGCGCGATTG
TCGGCACCAACGGCGGCGAGCTGCTGGGTGAGATCGGCCTGGCTATCGAGATGGGCTGTGACGCTGAA
GACATCGCCCTGACCATCCACGCTCACCCGACTCTGCACGAGTCCGTTGGCCTGGCGGCGGAAGTGTTC
GAAGGCAGCATCACCGACCTGCCAAACGCCAAAGCGAAGAAAAAGTAACTTTTTCTTTCAGGAAAAAAG
CATAAGCGGCTCCGGGAGCCGCTTTTTTTATGCCTGATGTTTAGAACTATGTCACTGTTCATAAACCGCTA
CACCTCATACATACTTTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATC
TAGCACATCCGGCAATTAAAAAAGCGGCTAACCACGCCGCTTTTTTTACGTCTGCAATTTACCTTTCCAGT
CTTCTTGCTCCACGTTCAGAGAGACGTTCGCATACTGCTGACCGTTGCTCGTTATTCAGCCTGACAGTAT
GGTTACTGTCGTTTAGACGTTGTGGGCGGCTCTCCTGAACTTTCTCCCGAAAAACCTGACGTTGTTCAGG
TGATGCCGATTGAACACGCTGGCGGGCGTTATCACGTTGCTGTTGATTCAGTGGGCGCTGCTGTACTTTT
TCCTTAAACACCTGGCGCTGCTCTGGTGATGCGGACTGAATACGCTCACGCGCTGCGTCTCTTCGCTGCT
GGTTCTGCGGGTTAGTCTGCATTTTCTCGCGAACCGCCTGGCGCTGCTCAGGCGAGGCGGACTGAATGC
GCTCACGCGCTGCCTCTCTTCGCTGCTGGATCTTCGGGTTAGTCTGCATTCTCTCGCGAACTGCCTGGCG
CTGCTCAGGCGAGGCGGACTGATAACGCTGACGAGCGGCGTCCTTTTGTTGCTGGGTCAGTGGTTGGC
GACGGCTGAAGTCGTGGAAGTCGTCATAGCTCCCATAGTGTTCAGCTTCATTAAACCGCTGTGCCGCTGC
CTGACGTTGGGTACCTCGTGTAATGACTGGTGCGGCGTGTGTTCGTTGCTGAAACTGATTTGCTGCCGCC
TGACGCTGGCTGTCGCGCGTTGGGGCAGGTAATTGCGTGGCGCTCATTCCGCCGTTGACATCGGTTTGA
TGAAACCGCTTTGCCATATCCTGATCATGATAGGGCACACCATTACGGTAGTTTGGATTGTGCCGCCATG
CCATATTCTTATCAGTAAGATGCTCACCGGTGATACGGTTGAAATTGTTGACGTCGATATTGATGTTGTC
GCCGTTGTGTTGCCAGCCATTACCGTCACGATGACCGCCATCGTGGTGATGATAATCAT

FIGURE 39 (cont'd)

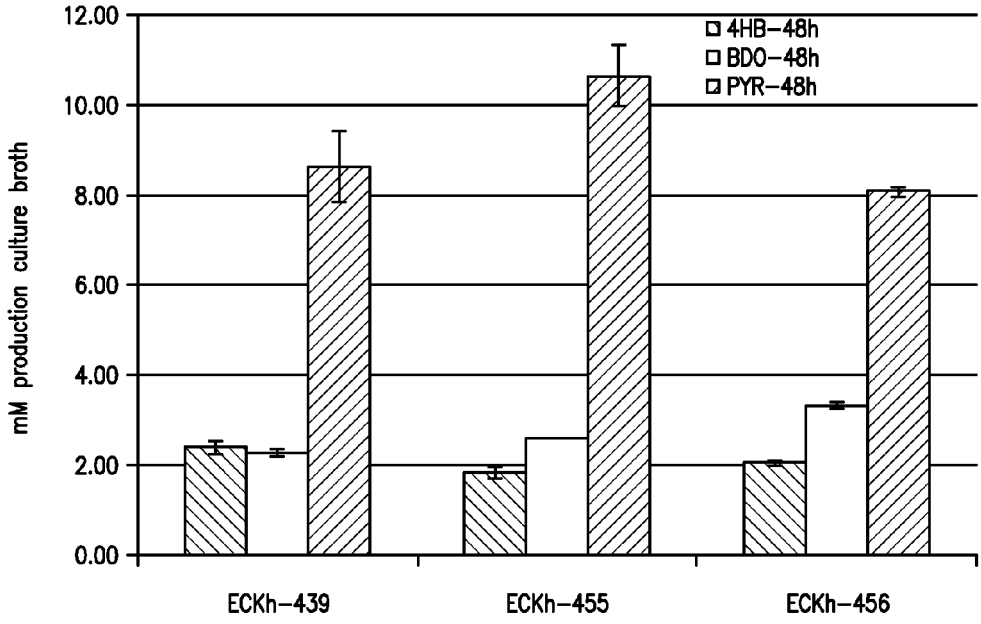


FIG. 40

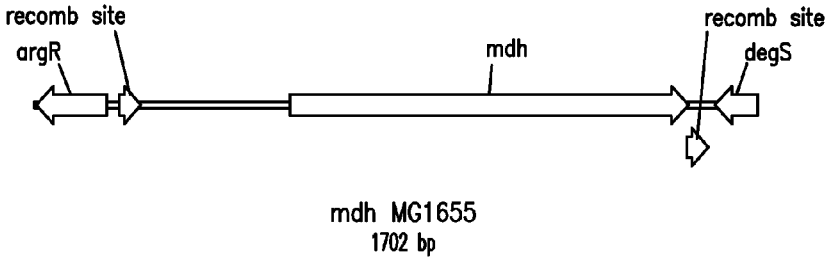


FIG. 41A


FRT
AS-mdh-Kan
XbaI
Cat
MetGluLys LysIleThr GlyTyrThrThr ValAspIle SerGlnIrp HisArgLysGlu HisPheGlu AlaPheGln
SerValAlaGln CysThrTyr AsnGlnThr ValGlnLeuAsp IleThrAla PheLeuLys ThrValLysLys AsnLysHis LysPheTyr ProAlaPheIle·
·IHisIleLeu AlaArgLeu MetAsnAlaHis ProGluLeu ArgMetAla MetLysAspGly GluLeuVal IleTrpAsp SerValHisPro CysTyrThr·
·ValPheHis GluGlnThrGlu ThrPheSer SerLeuTrp SerGluTyrHis AspAspPhe ArgGlnPhe LeuHisIleTyr SerGlnAsp ValAlaCys
TyrGlyGluAsn LeuAlaTyr PheProLys GlyPheIleGlu AsnMetPhe PheValSer AlaAsnProTrp ValSerPhe ThrSerPhe AspLeuAsnVal·
·VAlaAsnMet AspAsnPhe PheAlaProVal PheThrMet GlyLysTyr TyrThrGlnGly AspLysVal LeuMetPro LeuAlaIleGln ValHisHis·
·AlaValCys AspGlyPheHis ValGlyArg CysLeuMet AsnThrThrVal LeuArg** *
FRT
S-mdh-Kan

FIG. 41B

TTATTTGGTGATATTGGTACCAATATCATGCAGCAAACGGTGCAACATTGCCGTGTCTCGTTGCTCTAAA
AGCCCCAGGCGTTGTTGTAACCAGTCGACCAGTTTTATGTCATCTGCCACTGCCAGAGTCGTCAGCAATG
TCATGGCTCGTTCGCGTAAAGCTTGCAGTTGATGTTGGTCTGCCGTTGCATCACTTTTCGCCGGTTGTTGT
ATTAATGTTGCTAATTGATAGCAATAGACCATCACCGCCTGCCCCAGATTGAGCGAAGGATAATCCGCCA
CCATCGGCACACCAGTAAGAACGTCAGCCAACGCTAACTCTTCGTTAGTCAACCCGGAATCTTCGCGACC
AAACACCAGCGCGGCATGGCTCATCCATGAAGATTTTTCCTCTAACAGCGGCACCAGTTCAACTGGCGT
GGCGTAGTAATGATATTTCGCCCGACTGCGCGCAGTGGTGGCGACAGTGAAATCGACATCGTGTAACG
ATTCAGCCAATGTCGGGAAAACTTTAATATTATCAATAATATCACCAGATCCATGTGCGACCCAGCGGGT
GGCTGGCTCCAGGTGTGCCTGACTATCGACAATCCGCAGATCGCTAAACCCCATCGTTTTCATTGCCCGC
GCCGCTGCCCCAATATTTTCTGCTCTGGCGGGTGCGACCAGAATAATCGTTATACGCATATTGCCACTCTT
CTTGATCAAATAACCGCGAACCGGGTGATCACTGTCAACTTATTACGCGGTGCGAATTTACAAATTCTTA
ACGTAAGTCGCAGAAAAAGCCCTTTACTTAGCTTAAAAAAGGCTAAACTATTTCCTGACTGTACTAACGG
TTGAGTTGTTAAAAAATGCTACATATCCTTCTGTTTACTTAGGATAATTTTATAAAAAATAAATCTCGACA
ATTGGATTCACCACGTTTATTAGTTGTATGATGCAACTAGTTGGATTATTAAAATAATGTGACGAAAGCT
AGCATTTAGATACGATGATTTCATCAAACTGTTAACGTGCTACAATTGAACTTGATATATGTCAACGAAG
CGTAGTTTTATTGGGTGTCCGGCCCCTCTTAGCCTGTTATGTTGCTGTTAAAATGGTTAGGATGACAGCC
GTTTTTGACACTGTCGGGTCCTGAGGGAAAGTACCCACGACCAAGCTAATGATGTTGTTGACGTTGATG
GAAAGTGCATCAAGAACGCAATTACGTACTTTAGTCATGTTACGCCGATCATGTTAATTTGCAGCATGCA
TCAGGCAGGTCAGGGACTTTTGTACTTCCTGTTTCGATTTAGTTGGCAATTTAGGTAGCAAACGAATTCA
TCGGCTTTACCACCGTCAAAAAAAACGGCGCTTTTTAGCGCCGTTTTTATTTTTCAACCTTATTTCCAGATA
CGTAACTCATCGTCCGTTGTAACTTCTTTACTGGCTTTCATTTTCGGCAGTGAAAACGCATACCAGTCGAT
ATTACGGGTCACAAACATCATGCCGGCCAGCGCCACCACCAGCACACTGGTTCCCAACAACAGCGCGCT
ATCGGCAGAGTTGAGCAGTCCCCACATCACACCATCCAGCAACAACAGCGCGAGGGTAAACAACATGCT
GTTGCACCAACCTTTCAATACCGCTTGCAAATAAATACCGTTCATTATCGCCCCAATCAGACTGGCGATTA
TCCATGCCACGGTAAAACCGGTATGTTCAGAAAGCGCCAGCAAGAGCAAATAAAACATCACCAATGAAA
GCCCCACCAGCAAATATTGCATTGGGTGTAAACGTTGCGCGGTGAGCGTTTCAAAAACAAAGAACGCCA
TAAAAGTCAGTGCAATCAGCAGAATGGCGTACTTAGTCGCCCGGTCAGTTAATTGGTATTGATCGGCTG
GCGTCGTTACTGCGACGCTAAACGCCGGGAAGTTTTCCCAGCCGGTATCATTGCCTGAAGCAAAACGCT
CACCGAGATTATTAGCAAACCAGCTGCTTTGCCAGTGCGCCTGAAAACCTGACTCGCTAACTTCCCGTTT
GGCTGGTAGAAAATCACCTAAAAAACTGGGATGCGGCCAGTTGCTGGTTAAGGTCATTTCGCTATTACG
CCCGCCAGGCACCACAGAAAGATCGCCGGTACCGCTTAAATTCAGGGCCATATTCAGCTTCAGGTTCTG
CTTCCGCCAGTCCCCTTCAGGTAAAGGGATATGCACGCCCTGCCCGCCTTGCTCTAACCCGGTGCCGGGT
TCAATGGTCAGCGCCGTTCCGTTAACTTCAGGCGCTTTCACCACACCAATACCACGCGCATCCCCGACGC
TAATCACAATAAATGGCTTGCCTAAGGTGATATTTGGCGCGTTGAGTTCGCTAAGACGCGAAACATCGA
AATCGGCTTTTAACGTTAAATCACTGTGCCAGACCTGACCGGTATAAATCCCTATCTTGCGTTCTTCCACG
TTCTGATTGCCATCAACCATCAATGACTCAGGTAACCAAAAATGGATAAAACTTCGTTTCCGCTGCAGGG
TTTTAT

FIGURE 42

AAGCCACAGCAGGATGCCCACTGCAACAAAGGTGATCACACCGGAAACGCGATGGAGAATGGACGCTA
TCGCCGTGATGGGGAACCGGATGGTCTGTAGGTCCAGATTAACAGGTCTTTGTTTTTTCACATTTCTTAT
CATGAATAACGCCCACATGCTGTTCTTATTATTCCCTGGGGACTACGGGCACAGAGGTTAACTTTCTGTT
ACCTGGAGACGTCGGGATTTCCTTCCTCCGGTCTGCTTGCGGGTCAGACAGCGTCCTTTCTATAACTGCG
CGTCATGCAAAACACTGCTTCCAGATGCGAAAACGACACGTTACAACGCTGGGTGGCTCGGGATTGCAG
GGTGTTCCGGAGACCTGGCGGCAGTATAGGCTGTTCACAAAATCATTACAATTAACCTACATATAGTTTG
TCGGGTTTTATCCTGAACAGTGATCCAGGTCACGATAACAACATTTATTTAATTTTTAATCATCTAATTTG
ACAATCATTCAACAAAGTTGTTACAAACATTACCAGGAAAAGCATATAATGCGTAAAAGTTATGAAGTC
GGTATTTCACCTAAGATTAACTTATGTAACAGTGTGGAAGTATTGACCAATTCATTCGGGACAGTTATTA
GTGGTAGACAAGTTTAATAATTCGGATTGCTAAGTACTTGATTCGCCATTTATTCGTCATCAATGGATCCT
TTACCTGCAAGCGCCCAGAGCTCTGTACCCAGGTTTTCCCCTCTTTCACAGAGCGGCGAGCCAAATAAAA
AACGGGTAAAGCCAGGTTGATGTGCGAAGGCAAATTTAAGTTCCGGCAGTCTTACGCAATAAGGCGCT
AAGGAGACCTTAAATGGCTGATACAAAAGCAAAACTCACCCTCAACGGGGATACAGCTGTTGAACTGGA
TGTGCTGAAAGGCACGCTGGGTCAAGATGTTATTGATATCCGTACTCTCGGTTCAAAAGGTGTGTTCACC
TTTGACCCAGGCTTCACTTCAACCGCATCCTGCGAATCTAAAATTACTTTTATTGATGGTGATGAAGGTAT
TTTGCTGCACCGCGGTTTCCCGATCGATCAGCTGGCGACCGATTCTAACTACCTGGAAGTTTGTTACATC
CTGCTGAATGGTGAAAAACCGACTCAGGAACAGTATGACGAATTTAAAACTACGGTGACCCGTCATACC
ATGATCCACGAGCAGATTACCCGTCTGTTCCATGCTTTCCGTCGCGACTCGCATCCAATGGCAGTCATGT
GTGGTATTACCGGCGCGCTGGCGGCGTTCTATCACGACTCGCTGGATGTTAACAATCCTCGTCACCGTGA
AATTGCCGCGTTCCTCCTGCTGTCGAAAATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGGT
CAGCCATTTGTTTACCCGCGCAACGATCTCTCCTACGCCGGTAACTTCCTGAATATGATGTTCTCCACGCC
GTGCGAACCGTATGAAGTTAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGCTGAC
CATGAACAGAACGCCTCTACCTCCACCGTGCGTACCGCTGGCTCTTCGGGTGCGAACCCGTTTGCCTGTA
TCGCAGCAGGTATTGCTTCACTGTGGGGACCTGCGCACGGCGGTGCTAACGAAGCGGCGCTGAAAATG
CTGGAAGAAATCAGCTCCGTTAAACACATTCCGGAATTTGTTCGTCGTGCGAAAGACAAAAATGATTCTT
TCCGCCTGATGGGCTTCGGTCACCGCGTGTACAAAAATTACGACCCGCGCGCCACCGTAATGCGTGAAA
CCTGCCATGAAGTGCTGAAAGAGCTGGGCACGAAGGATGACCTGCTGGAAGTGGCTATGGAGCTGGAA
AACATCGCGCTGAACGACCCGTACTTTATCGAGAAGAAACTGTACCCGAACGTCGATTTCTACTCTGGTA
TCATCCTGAAAGCGATGGGTATTCCGTCTTCCATGTTCACCGTCATTTTCGCAATGGCACGTACCGTTGG
CTGGATCGCCCACTGGAGCGAAATGCACAGTGACGGTATGAAGATTGCCCGTCCGCGTCAGCTGTATAC
AGGATATGAAAAACGCGACTTTAAAAGCGATATCAAGCGTTAATGGTTGATTGCTAAGTTGTAAATATTT
TAACCCGCCGTTCATATGGCGGGTTGATTTTTATATGCCTAAACACAAAAAATTGTAAAAATAAAATCCA
TTAACAGACCTATATAGATATTTAAAAAGAATAGAACAGCTCAAATTATCAGCAACCCAATACTTTCAATT
AAAAACTTCATGGTAGTCGCATTTATAACCCTATGAAAATGACGTCTATCTATACCCCCCTATATTTTATTC
ATCATACAACAAATTCATGATACCAATAATTTAGTTTTGCATTTAATAAAACTAACAATATTTTTAAGCAA
AACTAAAAACTAGCAATAATCAAATACGATATTCTGGCGTAGCTATACCCCTATTCTATATCCTTAAAGGA
CTCTGTTATGTTTAAAGGACAAAAAACATTGGCCGCACTGGCCGTATCTCTGCTGTTCACTGCACCTGTTT
ATGCTGCTGATGAAGGTTCTGGCGAAATTCACTTTAAGGGGGAGGTTATTGAAGCACCTTGTGAAATTC
ATCCAGAAGATATTGATAAAAACATAGATCTTGGACAAGTCACGACAACCCATATAAACCGGGAGCATC
ATAGCAATAAAGTGGCCGTCGACATTCGCTTGATCAACTGTGATCTGCCTGCTTCTGACAACGGTAGCG
GAATGCCGGTATCCAAAGTTGGCGTAACCTTCGATAGCACGGCTAAGACAACTGGTGCTACGCCTTTGT
TGAGCAACACCAGTGCAGGCGAAGCAACTGGGGTCGGTGTACGACTGATGGACAAAAATGACGGTAAC
ATCGTATTAGGTTCAGCCGCGCCAGATCTTGACCTGGATGCAAGCTCATCAGAACAGACGCTGAACTTTT
TCGCCTGGAT

FIGURE 43

A. B.

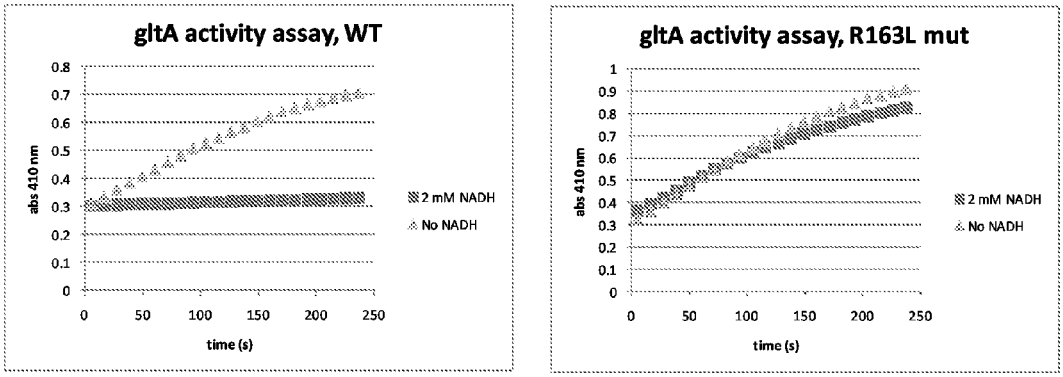


FIGURE 44

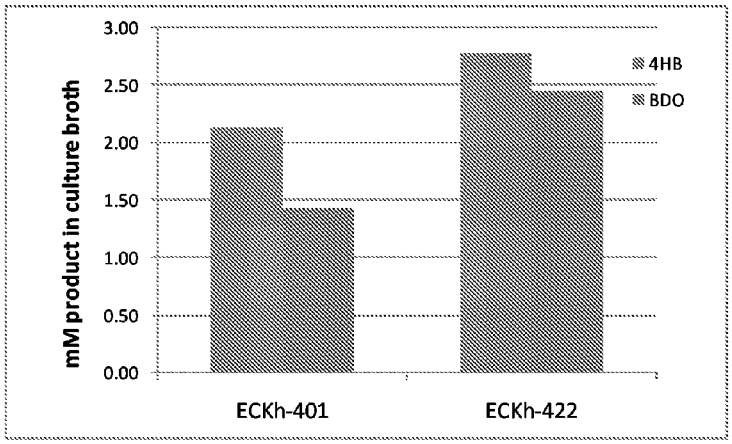


FIGURE 45

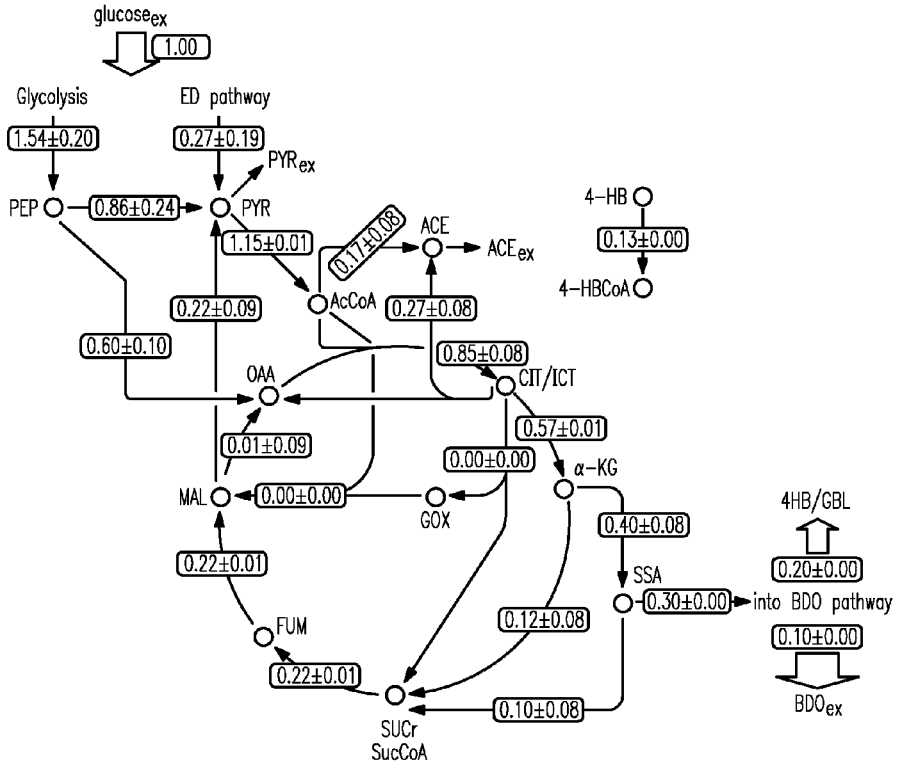


FIG. 46

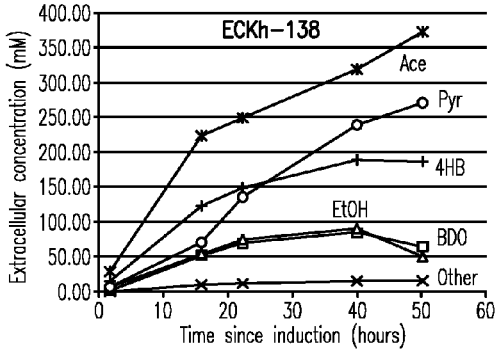


FIG. 47A

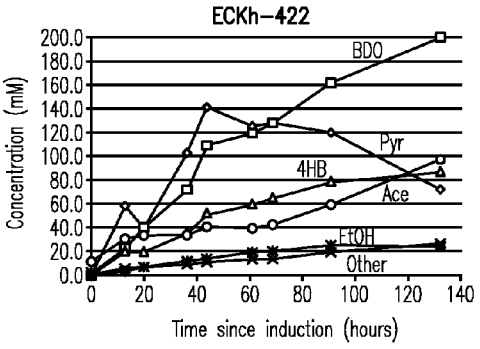


FIG. 47B

CGCGATGTCGACGTCACGAAACTGAAAAAACCGCTCTACATTCTGGCGACTGCTGATGAAGAAACCAGT
ATGGCCGGAGCGCGTTATTTTGCCGAAACTACCGCCCTGCGCCCGGATTGCGCCATCATTGGCGAACCG
ACGTCACTACAACCGGTACGCGCACATAAAGGTCATATCTCTAACGCCATCCGTATTCAGGGCCAGTCG
GGGCACTCCAGCGATCCAGCACGCGGAGTTAACGCTATCGAACTAATGCACGACGCCATCGGGCATATT
TTGCAATTGCGCGATAACCTGAAAGAACGTTATCACTACGAAGCGTTTACCGTGCCATACCCTACGCTCA
ACCTCGGGCATATTCACGGTGGCGACGCTTCTAACCGTATTTGCGCTTGCTGTGAGTTGCATATGGATAT
TCGTCCGCTGCCTGGCATGACACTCAATGAACTTAATGGTTTGCTCAACGATGCATTGGCTCCGGTGAGC
GAACGCTGGCCGGGTCGTCTGACGGTCGACGAGCTGCATCCGCCGATCCCTGGCTATGAATGCCCACCG
AATCATCAACTGGTTGAAGTGGTTGAGAAATTGCTCGGAGCAAAAACCGAAGTGGTGAACTACTGTACC
GAAGCGCCGTTTATTCAAACGTTATGCCCGACGCTGGTGTTGGGGCCTGGCTCAATTAATCAGGCTCATC
AACCTGATGAATATCTGGAAACACGGTTTATCAAGCCCACCCGCGAACTGATAACCCAGGTAATTCACCA
TTTTTGCTGGCATTAAAACGTAGGCCGGATAAGGCGCTCGCGCCGCATCCGGCGCTGTTGCCAAACTCC
AGTGCCGCAATAATGTCGGATGCGATGCTTGCGCATCTTATCCGACCTACAGTGACTCAAACGATGCCCA
ACCGTAGGCCGGATAAGGCGCTCGCGCCGCATCCGGCACTGTTGCCAAACTCCAGTGCCGCAATAATGT
CGGATGCGATACTTGCGCATCTTATCCGACCGACAGTGACTCAAACGATGCCCAACTGTAGGCCGGATA
AGGCGCTCGCGCCGCATCCGGCACTGTTGCCAAACTCCAGTGCCGCAATAATGTCGGATGCGATACTTG
CGCATCTTATCCGACCTACACCTTTGGTGTTACTTGGGGCGATTTTTTAACATTTCCATAAGTTACGCTTAT
TTAAAGCGTCGTGAATTTAATGACGTAAATTCCTGCTATTTATTCGTTTGCTGAAGCGATTTCGCAGCATT
TGACGTCACCGCTTTTACGTGGCTTTATAAAAGACGACGAAAAGCAAAGCCCGAGCATATTCGCGCCAA
TGCTAGCAAGAGGAGAAGTCGACATGACAGACTTAAATAAAGTGGTAAAAGAACTTGAAGCTCTTGGT
ATTTATGACGTAAAAGAAGTTGTTTACAATCCAAGCTACGAGCAATTGTTCGAAGAAGAAACTAAACCA
GGTTTAGAAGGCTTTGAAAAAGGTACTTTAACTACGACTGGTGCAGTGGCAGTAGATACAGGTATCTTC
ACAGGTCGTTCTCCAAAAGATAAATATATCGTGTTAGATGAAAAAACCAAAGATACTGTTTGGTGGACA
TCTGAAACAGCAAAAAACGACAACAAGCCAATGAACCAAGCTACATGGCAAAGCTTAAAAGACTTGGTA
ACCAACCAGCTTTCTCGTAAACGCTTATTTGTAGTTGATGGTTTCTGTGGTGCGAGCGAACACGACCGTA
TTGCAGTACGTATTGTCACTGAAGTAGCGTGGCAAGCACATTTTGTAAAAAATATGTTTATTCGCCCAAC
TGAAGAACAACTCAAAAATTTTGAACCAGATTTCGTTGTAATGAATGGTTCTAAAGTAACCAATCCAAAC
TGGAAAGAACAAGGTTTAAATTCAGAAAACTTTGTTGCTTTCAACTTGACTGAACGCATTCAATTAATCG
GTGGTACTTGGTACGGCGGTGAAATGAAAAAAGGTATGTTCTCAATCATGAACTACTTCCTACCACTTAA
AGGTGTTGGTGCAATGCACTGCTCAGCTAACGTTGGTAAAGATGGCGATGTAGCAATCTTCTTCGGCTT
ATCTGGCACAGGTAAAACAACCCTTTCAACGGATCCAAAACGTGAATTAATCGGTGACGATGAACACGG
CTGGGATGATGTGGGTATCTTTAACTTTGAAGGTGGTTGCTATGCGAAAACCATTCACCTTTCAGAAGAA
AATGAACCAGATATTTACCGCGCTATCCGTCGCGACGCATTATTAGAAAACGTGGTTGTTCGTGCAGATG
GTTCTGTTGATTTCGATGATGGTTCAAAAACAGAAAATACTCGCGTGTCTTACCCAATTTATCACATTGAT
AACATTGTAAAACCAGTTTCTCGTGCAGGTCACGCAACTAAAGTGATTTTCTTAACTGCAGATGCATTTG
GCGTATTACCACCAGTATCTAAATTGACACCAGAACAAACTAAATACTACTTCTTATCTGGTTTCACAGCA
AAATTAGCAGGTACTGAACGTGGTATTACTGAACCAACTCCAACTTTCTCAGCATGTTTCGGTGCTGCGT
TCTTAACCCTTCACCCAACTCAATATGCAGAAGTGTTAGTAAAACGTATGCAAGCAGTGGGTGCTGAAG
CTTACTTAGTAAATACTGGTTGGAATGGCACAGGCAAACGTATCTCAATCAAAGATACTCGCGGAATCAT
TGATGCAATCTTAGATGGCTCAATTGAAAAAGCTGAAATGGGCGAATTACCAATCTTTAACTTAGCCATT
CCTAAAGCATTACCAGGTGTAGATTCTGCAATCTTAGATCCTCGCGATACTTACGCAGATAAAGCACAAT
GGCAATCAAAAGCTGAAGACTTAGCAGGTCGTTTTGTGAAAAACTTTGTTAAATATGCAACTAACGAAG
AAGGCAAAGCTTTAATTGCAGCTGGTCCTAAAGCTTAATCTAGAAAGCTTCCTAGAGGCATCAAATAAA
ACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGT

FIGURE 48

AGGACGAATTCACTTCTGTTCTAACACCCTCGTTTTCAATATATTTCTGTCTGCATTTTATTCAAATTCTGA
ATATACCTTCAGATATCCTTAAGGAATTGTCGTTACATTCGGCGATATTTTTTCAAGACAGGTTCTTACTA
TGCATTCCACAGAAGTCCAGGCTAAACCTCTTTTTAGCTGGAAAGCCCTGGGTTGGGCACTGCTCTACTT
TTGGTTTTTCTCTACTCTGCTACAGGCCATTATTTACATCAGTGGTTATAGTGGCACTAACGGCATTCGCG
ACTCGCTGTTATTCAGTTCGCTGTGGTTGATCCCGGTATTCCTCTTTCCGAAGCGGATTAAAATTATTGCC
GCAGTAATCGGCGTGGTGCTATGGGCGGCCTCTCTGGCGGCGCTGTGCTACTACGTCATCTACGGTCAG
GAGTTCTCGCAGAGCGTTCTGTTTGTGATGTTCGAAACCAACACCAACGAAGCCAGCGAGTATTTAAGC
CAGTATTTCAGCCTGAAAATTGTGCTTATCGCGCTGGCCTATACGGCGGTGGCAGTTCTGCTGTGGACAC
GCCTGCGCCCGGTCTATATTCCAAAGCCGTGGCGTTATGTTGTCTCTTTTGCCCTGCTTTATGGCTTGATT
CTGCATCCGATCGCCATGAATACGTTTATCAAAAACAAGCCGTTTGAGAAAACGTTGGATAACCTGGCCT
CGCGTATGGAGCCTGCCGCACCGTGGCAATTCCTGACCGGCTATTATCAGTATCGTCAGCAACTAAACTC
GCTAACAAAGTTACTGAATGAAAATAATGCCTTGCCGCCACTGGCTAATTTCAAAGATGAATCGGGTAA
CGAACCGCGCACTTTAGTGCTGGTGATTGGCGAGTCGACCCAGCGCGGACGCATGAGTCTGTACGGTTA
TCCGCGTGAAACCACGCCGGAGCTGGATGCGCTGCATAAAACCGATCCGAATCTGACCGTGTTTAATAA
CGTAGTTACGTCTCGTCCGTACACCATTGAAATCCTGCAACAGGCGCTGACCTTTGCCAATGAAAAGAAC
CCGGATCTGTATCTGACGCAGCCGTCGCTGATGAACATGATGAAACAGGCGGGTTATAAAACCTTC

FIGURE 48 (cont'd)

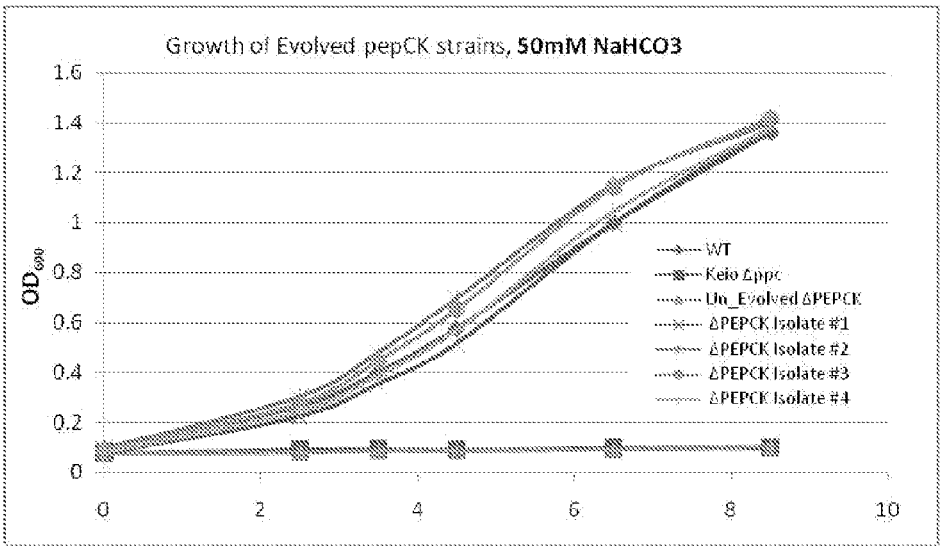


FIGURE 49

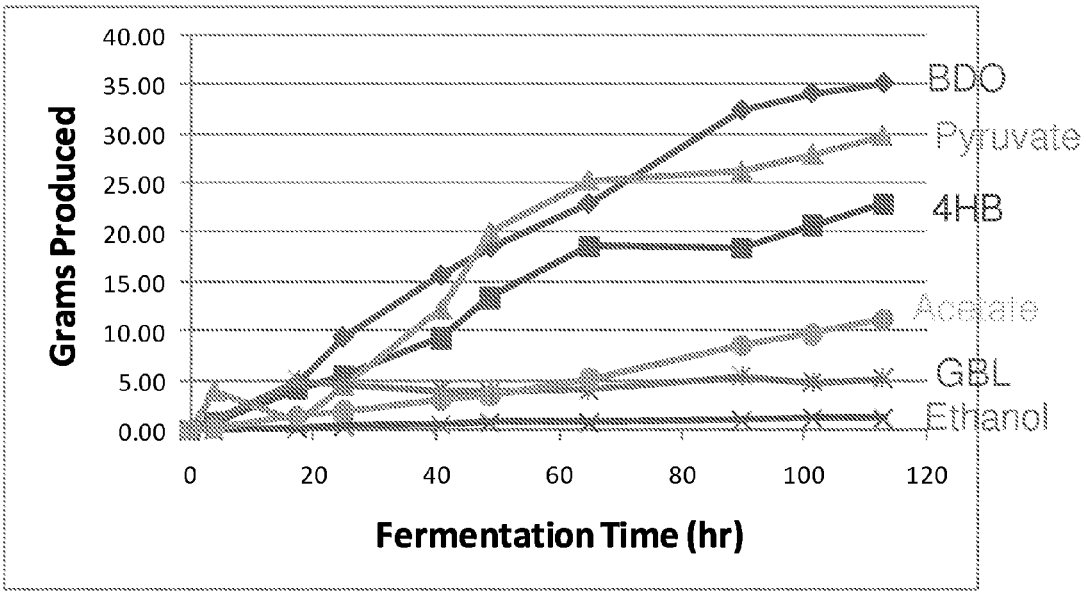


FIGURE 50

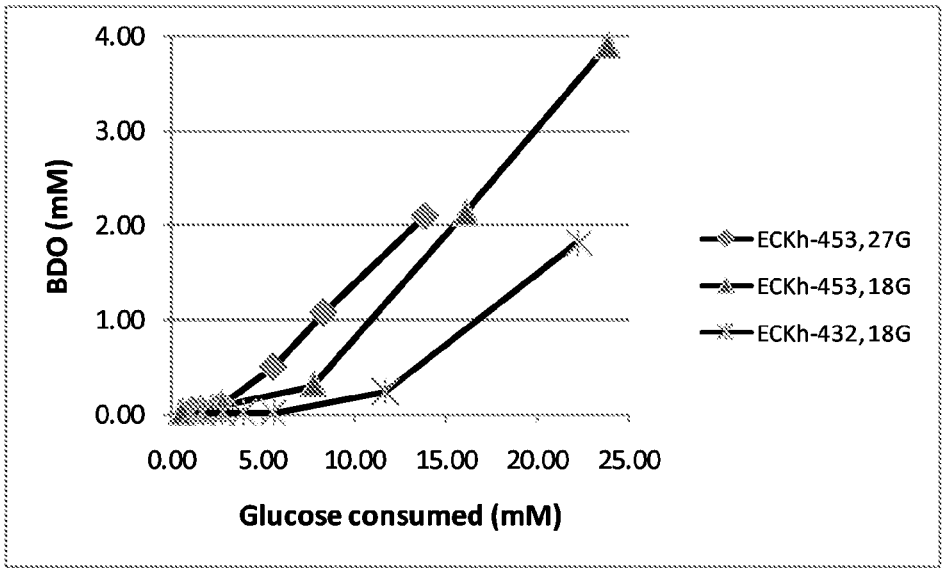

4.00
3.00
2.00
1.00
0.00
BDO (mM)
0.00
5.00
10.00
15.00
20.00
25.00
Glucose consumed (mM)
ECKh-453,27G
ECKh-453,18G
ECKh-432,18G

FIGURE 51

AATAGGCGTATCACGAGGCCCTTTCGTCTTCACCTCGAGAATTGTGAGCGGATAACAATTGACATTGTGA
GCGGATAACAAGATACTGAGCACATCAGCAGGACGCACTGACCGAATTCAATTAAGCTAGCAAGAGGA
GAAGTCGAGATGAACTTACATGAATATCAGGCAAAACAACTTTTTGCCCGCTATGGCTTACCAGCACCG
GTGGGTTATGCCTGTACTACTCCGCGCGAAGCAGAAGAAGCCGCTTCAAAAATCGGTGCCGGTCCGTGG
GTAGTGAAATGTCAGGTTCACGCTGGTGGCCGCGGTAAAGCGGGCGGTGTGAAAGTTGTAAACAGCAA
AGAAGACATCCGTGCTTTTGCAGAAAACTGGCTGGGCAAGCGTCTGGTAACGTATCAAACAGATGCCAA
TGGCCAACCGGTTAACCAGATTCTGGTTGAAGCAGCGACCGATATCGCTAAAGAGCTGTATCTCGGTGC
CGTTGTTGACCGTAGTTCCCGTCGTGTGGTCTTTATGGCCTCCACCGAAGGCGGCGTGGAAATCGAAAA
AGTGGCGGAAGAAACTCCGCACCTGATCCATAAAGTTGCGCTTGATCCGCTGACTGGCCCGATGCCGTA
TCAGGGACGCGAGCTGGCGTTCAAACTGGGTCTGGAAGGTAAACTGGTTCAGCAGTTCACCAAAATCTT
CATGGGCCTGGCGACCATTTTCCTGGAGCGCGACCTGGCGTTGATCGAAATCAACCCGCTGGTCATCAC
CAAACAGGGCGATCTGATTTGCCTCGACGGCAAACTGGGCGCTGACGGCAACGCACTGTTCCGCCAGCC
TGATCTGCGCGAAATGCGTGACCAGTCGCAGGAAGATCCGCGTGAAGCACAGGCTGCACAGTGGGAAC
TGAACTACGTTGCGCTGGACGGTAACATCGGTTGTATGGTTAACGGCGCAGGTCTGGCGATGGGTACG
ATGGACATCGTTAAACTGCACGGCGGCGAACCGGCTAACTTCCTTGACGTTGGCGGCGGCGCAACCAAA
GAACGTGTAACCGAAGCGTTCAAAATCATCCTCTCTGACGACAAAGTGAAAGCCGTTCTGGTTAACATCT
TCGGCGGTATCGTTCGTTGCGACCTGATCGCTGACGGTATCATCGGCGCGGTAGCAGAAGTGGGTGTTA
ACGTACCGGTCGTGGTACGTCTGGAAGGTAACAACGCCGAACTCGGCGCGAAGAAACTGGCTGACAGC
GGCCTGAATATTATTGCAGCAAAAGGTCTGACGGATGCAGCTCAGCAGGTTGTTGCCGCAGTGGAGGG
GAAATAATGTCCATTTTAATCGATAAAAACACCAAGGTTATCTGCCAGGGCTTTACCGGTAGCCAGGGG
ACTTTCCACTCAGAACAGGCCATTGCATACGGCACTAAAATGGTTGGCGGCGTAACCCCAGGTAAAGGC
GGCACCACCCACCTCGGCCTGCCGGTGTTCAACACCGTGCGTGAAGCCGTTGCTGCCACTGGCGCTACC
GCTTCTGTTATCTACGTACCAGCACCGTTCTGCAAAGACTCCATTCTGGAAGCCATCGACGCAGGCATCA
AACTGATTATCACCATCACTGAAGGCATCCCGACGCTGGATATGCTGACCGTGAAAGTGAAGCTGGATG
AAGCAGGCGTTCGTATGATCGGCCCGAACTGCCCAGGCGTTATCACTCCGGGTGAATGCAAAATCGGTA
TCCAGCCTGGTCACATTCACAAACCGGGTAAAGTGGGTATCGTTTCCCGTTCCGGTACACTGACCTATGA
AGCGGTTAAACAGACCACGGATTACGGTTTCGGTCAGTCGACCTGTGTCGGTATCGGCGGTGACCCGAT
CCCGGGCTCTAACTTTATCGACATTCTCGAAATGTTCGAAAAAGATCCGCAGACCGAAGCGATCGTGAT
GATCGGTGAGATCGGCGGTAGCGCTGAAGAAGAAGCAGCTGCGTACATCAAAGAGCACGTTACCAAGC
CAGTTGTGGGTTACATCGCTGGTGTGACTGCGCCGAAAGGCAAACGTATGGGCCACGCGGGTGCCATC
ATTGCCGGTGGGAAAGGGACTGCGGATGAGAAATTCGCTGCTCTGGAAGCCGCAGGCGTGAAAACCGT
TCGCAGCCTGGCGGATATCGGTGAAGCACTGAAAACTGTTCTGAAATAATCTAGCAAGAGGAGAAGTC
GACATGGAAATCAAAGAAATGGTGAGCCTTGCACGCAAGGCTCAGAAGGAGTATCAAGCTACCCATAA
CCAAGAAGCAGTTGACAACATTTGCCGAGCTGCAGCAAAAGTTATTTATGAAAATGCAGCTATTCTGGC
TCGCGAAGCAGTAGACGAAACCGGCATGGGCGTTTACGAACACAAAGTGGCCAAGAATCAAGGCAAAT
CCAAAGGTGTTTGGTACAACCTCCACAATAAAAAATCGATTGGTATCCTCAATATAGACGAGCGTACCG
GTATGATCGAGATTGCAAAGCCTATCGGAGTTGTAGGAGCCGTAACGCCGACGACCAACCCGATCGTTA
CTCCGATGAGCAATATCATCTTTGCTCTTAAGACCTGCAATGCCATCATTATTGCCCCCCACCCCAGATCC
AAAAAATGCTCTGCACACGCAGTTCGTCTGATCAAAGAAGCTATCGCTCCGTTCAACGTACCGGAAGGT
ATGGTTCAGATCATCGAAGAACCCAGCATCGAGAAGACGCAGGAACTCATGGGCGCCGTAGACGTAGT
AGTTGCTACGGGTGGTATGGGCATGGTGAAGTCTGCATATTCTTCAGGAAAGCCTTCTTTCGGTGTTGG
AGCCGGTAACGTTCAGGTGATCGTGGATAGCAACATCGATTTCGAAGCTGCTGCAGAAAAAATCATCAC

FIGURE 52

CGGTCGTGCTTTCGACAACGGTATCATCTGCTCAGGCGAACAGAGCATCATCTACAACGAGGCTGACAA
GGAAGCAGTTTTCACAGCATTCCGCAACCACGGTGCATATTTCTGTGACGAAGCCGAAGGAGATCGGGC
TCGTGCAGCTATCTTCGAAAATGGAGCCATCGCGAAAGATGTAGTAGGTCAGAGCGTTGCCTTCATTGC
CAAGAAAGCAAACATCAATATCCCCGAGGGTACCCGTATTCTCGTTGTTGAAGCTCGCGGCGTAGGAGC
AGAAGACGTTATCTGTAAGGAAAAGATGTGTCCCGTAATGTGCGCCCTCAGCTACAAGCACTTCGAAGA
AGGTGTAGAAATCGCACGTACGAACCTCGCCAACGAAGGTAACGGCCACACCTGTGCTATCCACTCCAA
CAATCAGGCACACATCATCCTCGCAGGATCAGAGCTGACGGTATCTCGTATCGTAGTGAATGCTCCGAG
TGCCACTACAGCAGGCGGTCACATCCAAAACGGTCTTGCCGTAACCAATACGCTCGGATGCGGATCATG
GGGTAATAACTCTATCTCCGAGAACTTCACTTACAAGCACCTCCTCAACATTTCACGCATCGCACCGTTGA
ATTCAAGCATTCACATCCCCGATGACAAAGAAATCTGGGAACTCTAATCTAGCAAGAGGAGAAGTCGAC
ATGCAACTTTTCAAACTCAAGAGTGTAACACATCACTTTGACACTTTTGCAGAATTTGCCAAGGAATTCTG
TCTTGGAGAACGCGACTTGGTAATTACCAACGAGTTCATCTATGAACCGTATATGAAGGCATGCCAGCTC
CCCTGCCATTTTGTTATGCAGGAGAAATATGGGCAAGGCGAGCCTTCTGACGAAATGATGAATAACATC
TTGGCAGACATCCGTAATATCCAGTTCGACCGCGTAATCGGTATCGGAGGAGGTACGGTTATTGACATC
TCTAAACTTTTCGTTCTGAAAGGATTAAATGATGTACTCGATGCATTCGACCGCAAAATACCTCTTATCAA
AGAGAAAGAACTGATCATTGTGCCCACAACATGCGGAACGGGTAGCGAGGTGACGAACATTTCTATCG
CAGAAATCAAAAGCCGTCACACCAAAATGGGATTGGCTGACGATGCCATTGTTGCAGACCATGCCATCA
TCATACCTGAACTTCTGAAGAGCTTGCCTTTCCACTTCTACGCATGCAGTGCAATCGATGCTCTTATCCAT
GCCATCGAGTCATACGTATCTCCTAAAGCCAGTCCATATTCTCGTCTGTTCAGTGAGGCGGCTTGGGACA
TTATCCTGGAAGTATTCAAGAAAATCGCCGAACACGGCCCTGAATACCGCTTCGAAAAGCTGGGAGAAA
TGATCATGGCCAGCAACTATGCCGGTATAGCCTTCGGAAATGCAGGAGTAGGAGCCGTCCACGCACTAT
CCTACCCGTTGGGAGGCAACTATCACGTGCCGCATGGAGAAGCAAACTATCAGTTCTTCACAGAGGTAT
TCAAAGTATACCAAAAGAAGAATCCTTTCGGCTATATAGTCGAACTCAACTGGAAGCTCTCCAAGATACT
GAACTGCCAGCCCGAATACGTATATCCGAAGCTGGATGAACTTCTCGGATGCCTTCTTACCAAGAAACCT
TTGCACGAATACGGCATGAAGGACGAAGAGGTAAGAGGCTTTGCGGAATCAGTGCTTAAGACACAGCA
AAGATTGCTCGCCAACAACTACGTAGAGCTTACTGTAGATGAGATCGAAGGTATCTACAGAAGACTCTA
CTAATCTAGAAAGCTTCCTAGAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGT
TTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGCCCTAGACCTAGGCGTTCG
GCTGCGACACGTCTTGAGCGATTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAG
GAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGACCATGGCTAATTCCCAT

FIGURE 52 (cont'd)

TCGAGAAATTTATCAAAAAGAGTGTTGACTTGTGAGCGGATAACAATGATACTTAGATTCAATTGTGAG
CGGATAACAATTTCACACAGAATTCAATTAAGCTAGCAAGAGGAGAAGTCGACATGGCCAACATAAGTT
CACCATTCGGGCAAAACGAATGGCTGGTTGAAGAGATGTACCGCAAGTTCCGCGACGACCCCTCCTCGG
TCGATCCCAGCTGGCACGAGTTCCTGGTTGACTACAGCCCCGAACCCACCTCCCAACCAGCTGCCGAACC
AACCCGGGTTACCTCGCCACTCGTTGCCGAGCGGGCCGCTGCGGCCGCCCCGCAGGCACCCCCCAAGCC
GGCCGACACCGCGGCCGCGGGCAACGGCGTGGTCGCCGCACTGGCCGCCAAAACTGCCGTTCCCCCGC
CAGCCGAAGGTGACGAGGTAGCGGTGCTGCGCGGCGCCGCCGCGGCCGTCGTCAAGAACATGTCCGC
GTCGTTGGAGGTGCCGACGGCGACCAGCGTCCGGGCGGTCCCGGCCAAGCTACTGATCGACAACCGGA
TCGTCATCAACAACCAGTTGAAGCGGACCCGCGGCGGCAAGATCTCGTTCACGCATTTGCTGGGCTACG
CCCTGGTGCAGGCGGTGAAGAAATTCCCGAACATGAACCGGCACTACACCGAAGTCGACGGCAAGCCC
ACCGCGGTCACGCCGGCGCACACCAATCTCGGCCTGGCGATCGACCTGCAAGGCAAGGACGGGAAGCG
TTCCCTGGTGGTGGCCGGCATCAAGCGGTGCGAGACCATGCGATTCGCGCAGTTCGTCACGGCCTACGA
AGACATCGTACGCCGGGCCCGCGACGGCAAGCTGACCACTGAAGACTTTGCCGGCGTGACGATTTCGCT
GACCAATCCCGGAACCATCGGCACCGTGCATTCGGTGCCGCGGCTGATGCCCGGCCAGGGCGCCATCAT
CGGCGTGGGCGCCATGGAATACCCCGCCGAGTTTCAAGGCGCCAGCGAGGAACGCATCGCCGAGCTGG
GCATCGGCAAATTGATCACTTTGACCTCCACCTACGACCACCGCATCATCCAGGGCGCGGAATCGGGCG
ACTTCCTGCGCACCATCCACGAGTTGCTGCTCTCGGATGGCTTCTGGGACGAGGTCTTCCGCGAACTGAG
CATCCCATATCTGCCGGTGCGCTGGAGCACCGACAACCCCGACTCGATCGTCGACAAGAACGCTCGCGT
CATGAACTTGATCGCGGCCTACCGCAACCGCGGCCATCTGATGGCCGATACCGACCCGCTGCGGTTGGA
CAAAGCTCGGTTCCGCAGTCACCCCGACCTCGAAGTGCTGACCCACGGCCTGACGCTGTGGGATCTCGA
TCGGGTGTTCAAGGTCGACGGCTTTGCCGGTGCGCAGTACAAGAAACTGCGCGACGTGCTGGGCTTGCT
GCGCGATGCCTACTGCCGCCACATCGGCGTGGAGTACGCCCATATCCTCGACCCCGAACAAAAGGAGTG
GCTCGAACAACGGGTCGAGACCAAGCACGTCAAACCCACTGTGGCCCAACAGAAATACATCCTCAGCAA
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GTACTCGCAGATCTTCACCGAGTTCGAGGGCAACCTGAATCCGTCGCAGGCGCACGGCTCCGGTGACGT
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GACCGCCAACCCGTCGCATCTGGAGGCCGTCGACCCGGTGCTGGAGGGATTGGTGCGGGCCAAGCAGG
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AGATGATTCCCGCGGGGCTGGCCACTGCGGTGGACAAGTCGCTGCTGGCCCGGATCGGCGATGCGTTC
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GGCCTATGAAGGCAAGATCGACTGGGCCTTTGGCGAGCTGCTGGCGCTGGGCTCGCTGGTGGCCGAAG
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TCGACCGCCACACTGGCGAGGAGTTCACACCACTGCAGCTGCTGGCGACCAACTCCGACGGCAGCCCGA
CCGGCGGAAAGTTCCTGGTCTACGACTCGCCACTGTCGGAGTACGCCGCCGTCGGCTTCGAGTACGGCT
ACACTGTGGGCAATCCGGACGCCGTGGTGCTCTGGGAGGCGCAGTTCGGCGACTTCGTCAACGGCGCA

FIGURE 53

CAGTCGATCATCGACGAGTTCATCAGCTCCGGTGAGGCCAAGTGGGGCCAATTGTCCAACGTCGTGCTG
CTGTTACCGCACGGGCACGAGGGGCAGGGACCCGACCACACTTCTGCCCGGATCGAACGCTTCTTGCAG
TTGTGGGCGGAAGGTTCGATGACCATCGCGATGCCGTCGACTCCGTCGAACTACTTCCACCTGCTACGCC
GGCATGCCCTGGACGGCATCCAACGCCCGCTGATCGTGTTCACGCCCAAGTCGATGTTGCGTCACAAGG
CCGCCGTCAGCGAAATCAAGGACTTCACCGAGATCAAGTTCCGCTCAGTGCTGGAGGAACCCACCTATG
AGGACGGCATCGGAGACCGCAACAAGGTCAGCCGGATCCTGCTGACCAGTGGCAAGCTGTATTACGAG
CTGGCCGCCCGCAAGGCCAAGGACAACCGCAATGACCTCGCGATCGTGCGGCTTGAACAGCTCGCCCC
GCTGCCCAGGCGTCGACTGCGTGAAACGCTGGACCGCTACGAGAACGTCAAGGAGTTCTTCTGGGTCCA
AGAGGAACCGGCCAACCAGGGTGCGTGGCCGCGATTCGGGCTCGAACTACCCGAGCTGCTGCCTGACA
AGTTGGCCGGGATCAAGCGAATCTCGCGCCGGGCGATGTCAGCCCCGTCGTCAGGCTCGTCGAAGGTG
CACGCCGTCGAACAGCAGGAGATCCTCGACGAGGCGTTCGGCTAATCTAGCAAGAGGAGAAGTCGACA
TGAAGTTATTAAAATTGGCACCTGATGTTTATAAATTTGATACTGCAGAGGAGTTTATGAAATACTTTAA
GGTTGGAAAAGGTGACTTTATACTTACTAATGAATTTTTATATAAACCTTTCCTTGAGAAATTCAATGATG
GTGCAGATGCTGTATTTCAGGAGAAATATGGACTCGGTGAACCTTCTGATGAAATGATAAACAATATAA
TTAAGGATATTGGAGATAAACAATATAATAGAATTATTGCTGTAGGGGGAGGATCTGTAATAGATATAG
CCAAAATCCTCAGTCTTAAGTATACTGATGATTCATTGGATTTGTTTGAGGGAAAAGTACCTCTTGTAAA
AAACAAAGAATTAATTATAGTTCCAACTACATGTGGAACAGGTTCAGAAGTTACAAATGTATCAGTTGCA
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GTACCAGAATTTATAAAAGGACTTCCATATAAGTTTTTTGTAACCAGCTCCGTAGATGCCTTAATACATGC
AACAGAAGCTTATGTATCTCCAAATGCAAATCCTTATACTGATATGTTTAGTGTAAAAGCTATGGAGTTA
ATTTTAAATGGATACATGCAAATGGTAGAGAAAGGAAATGATTACAGAGTTGAAATAATTGAGGATTTT
GTTATAGGCAGCAATTATGCAGGTATAGCTTTTGGAAATGCAGGAGTGGGAGCGGTTCACGCACTCTCA
TATCCAATAGGCGGAAATTATCATGTGCCTCATGGAGAAGCAAATTATCTGTTTTTTACAGAAATATTTA
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AAGAGAATATGGAATGAAAGAGGAAGAAATTGAAACTTTTGCTGATTCAGTAATAGAAGGACAGCAGA
GACTGTTGGTAAACAATTATGAACCTTTTTCAAGAGAAGACATAGTAAACACATATAAAAAGTTATATTA
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FIGURE 53 (cont'd)

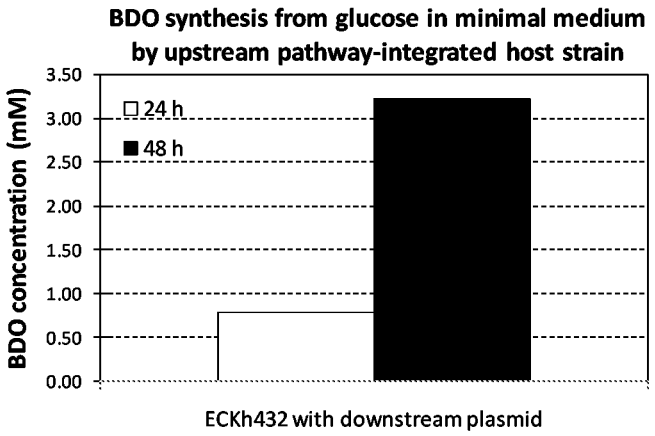

BDO synthesis from glucose in minimal medium
by upstream pathway-integrated host strain
BDO concentration (mM)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
24 h
48 h
ECKh432 with downstream plasmid

FIGURE 54

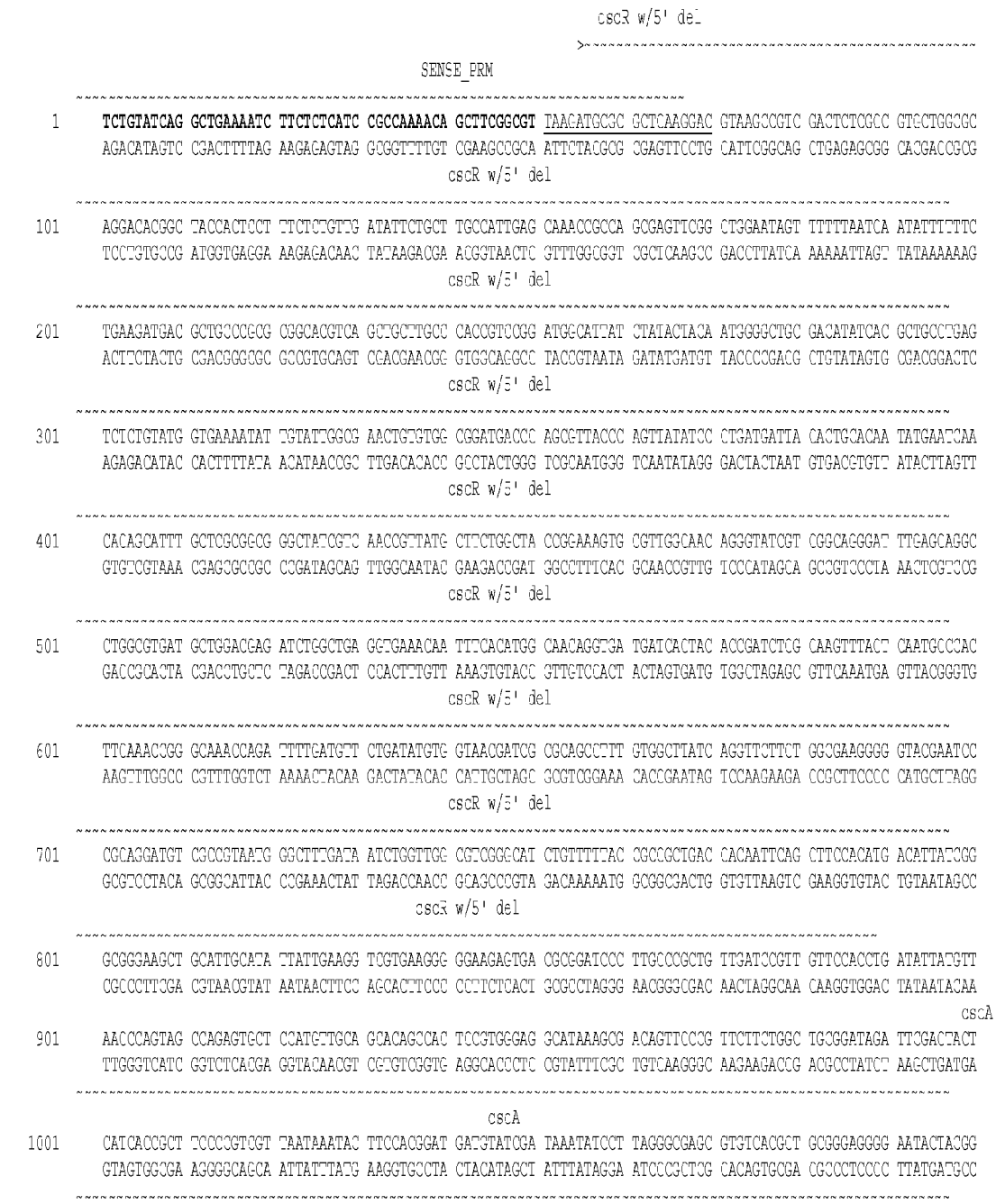
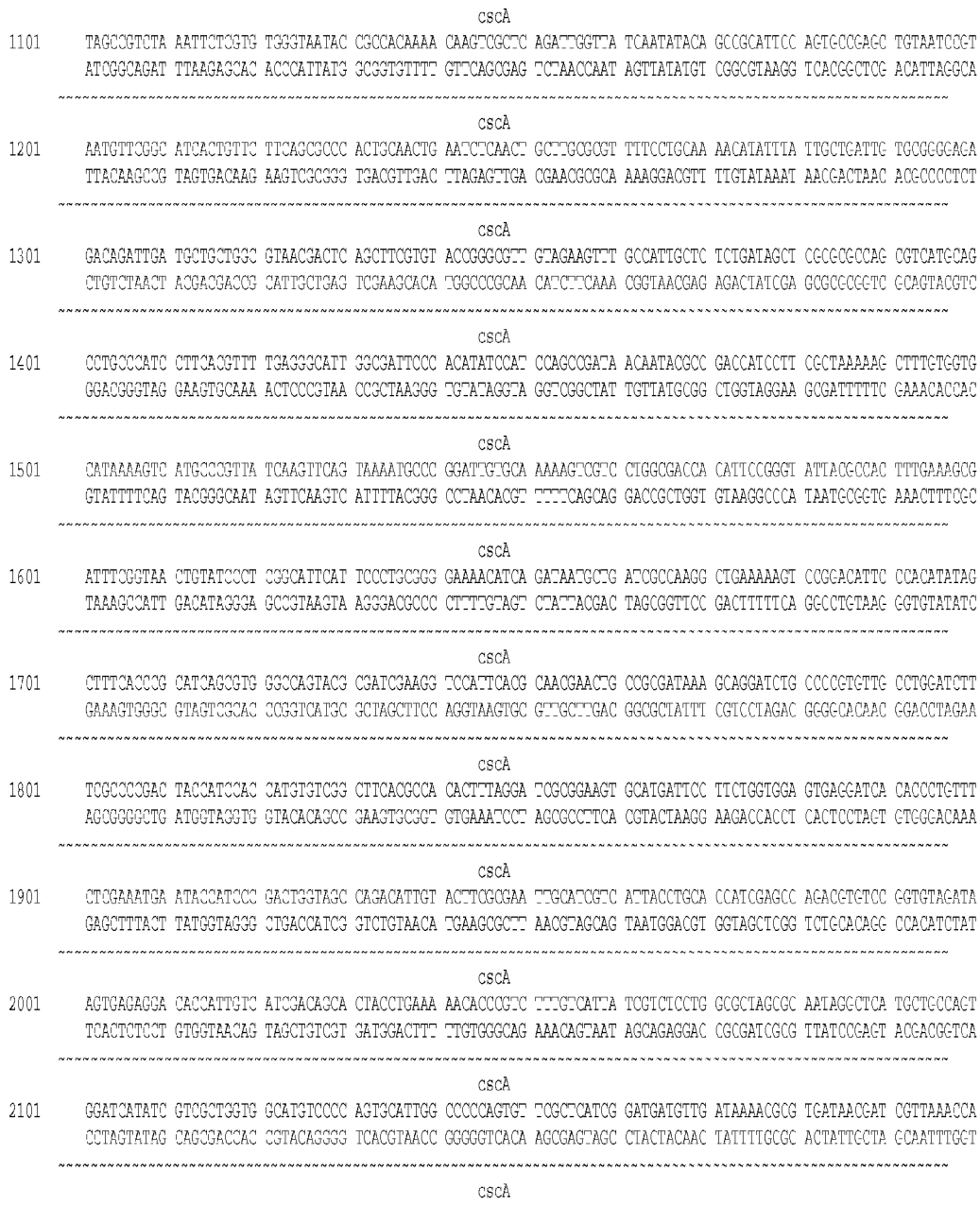

cscR w/5' del
SENSE_PRM
cscR w/5' del
cscR w/5' del
cscR w/5' del
cscR w/5' del
cscR w/5' del
cscR w/5' del
cscA
cscA

FIG. 55


cscA
1101
TAGCCGTCTA AATTGTCGTG TGGGTAATAC CGCCACAAAA CAAGTCGCTC AGATTGGTTA TCAATATACA GCCGCATTCC AGTGCCGAGC TGTAATCCGT
ATCGGCAGAT TTAAGAGCAC ACCCATTATG GCGGTGTTTT GTTCAGCGAG TCTAACCAAT AGTTATATGT CGGCGTAAGG TCACGGCTCG ACATTAGGCA
cscA
1201
AATGTTCGGC ATCACTGTTC TTCAGCGCCC ACTGCAACTG AATCTCAACT GCTTGCGCGT TTTCCTGCAA AACATATTTA TTGCTGATTC TGCGGCGCAGA
TTACAAGCCG TAGTGACAAG AAGTCGCGGG TGACGTTGAC TTAGAGTTGA CGAACGCGCA AAAGGACGTT TTGTATAAAT AACGACTAAC ACGCCCCTCT
cscA
1301
GACAGATTGA TGCTGCTGGC GTAACGACTC AGCTTCGTGT ACCGGGCGTT GTAGAAGTTT GCCATTGCTC TCTGATAGCT CGCGCGCCAG CGTCATGCAG
CTGTCTAACT ACGACGACCG CATTGCTGAG TCGAAGCACA TGGCCCGCAA CATCTTCAAA CGGTAACGAG AGACTATCGA GCGCGCGGTC GCAGTACGTC
cscA
1401
CCTGCCCATC CTTCACGTTT TGAGGGCATT GGCGATTCCC ACATATCCAT CCAGCCGATA ACAATACGCC GACCATCCTT CGCTAAAAAG CTTTGTGGTG
GGACGGGTAG GAAGTGCAAA ACTCCCGTAA CCGCTAAGGG TGTATAGGTA GGTCGGCTAT TGTTATGCGG CTGGTAGGAA GCGATTTTTC GAAACACCAC
cscA
1501
CATAAAAGTC ATGCCCGTTA TCAAGTTCAG TAAAATGCCC GGATTGTGCA AAAAGTCGTC CTGGCGACCA CATTCCGGGT ATTACGCCAC TTTGAAAGCG
GTATTTTCAG TACGGGCAAT AGTTCAAGTC ATTTTACGGG CCTAACACGT TTTTCAGCAG GACCGCTGGT GTAAGGCCCA TAATGCGGTG AAACTTTCGC
cscA
1601
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TAAAGCCATT GACATAGGGA GCCGTAAGTA AGGGACGCCC CTTTGTAGT CTATTACGAC TAGCGGTTCC GACTTTTTCA GGCCTGTAAG GGTGTATATC
cscA
1701
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GAAAGTGGGC GTAGTCGCAC CCGGTCATGC GCTAGCTTCC AGGTAAGTGC GTTGCTTGAC GGCGCTATTT CGTCCTAGAC GGGGCACAAC GGACCTAGAA
cscA
1801
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cscA
1901
CTCGAAATGA ATACCATCCC GACTGGTAGC CAGACATTGT ACTTCGCGAA TTGCATCGTC ATTACCTGCA CCATCGAGCC AGACGTGTCC GGTGTAGATA
GAGCTTTACT TATGGTAGGG CTGACCATCG GTCTGTAACA TGAAGCGCTT AACGTAGCAG TAATGGACGT GGTAGCTCGG TCTGCACAGG CCACATCTAT
cscA
2001
AGTGAGAGGA CACCATTGTC ATCGACAGCA CTACCTGAAA AACACCCGTC TTTGTCATTA TCGTCTCCTG GCGCTAGCGC AATAGGCTCA TGCTGCCAGT
TCACTCTCCT GTGGTAACAG TAGCTGTCGT GATGGACTTT CTGTGGGCAG AAACAGTAAT AGCAGAGGAC CGCGATCGCG TTATCCGAGT ACGACGGTCA
cscA
2101
GGATCATATC GTCGCTGGTG GCATGTCCCC AGTGCATTGG CCCCCAGTGT TCGCTCATCG GATGATGTTG ATAAAACGCG TGATAACGAT CGTTAAACCA
CCTAGTATAG CAGCGACCAC CGTACAGGGG TCACGTAACC GGGGGTCACA AGCGAGTAGC CTACTACAAC TATTTTGCGC ACTATTGCTA GCAATTTGGT
cscA

FIG. 55 (cont'd)

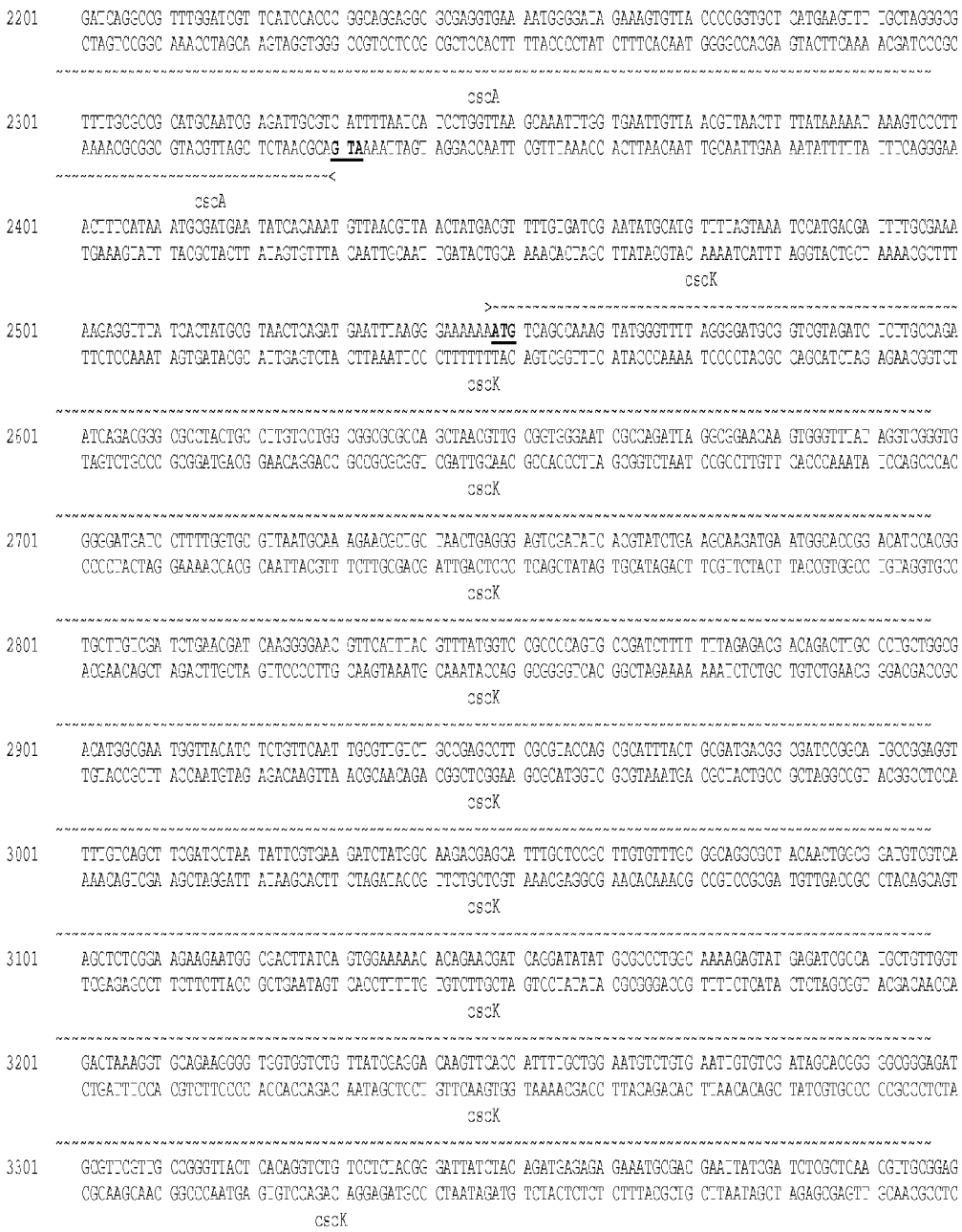


FIG. 55 (cont'd)

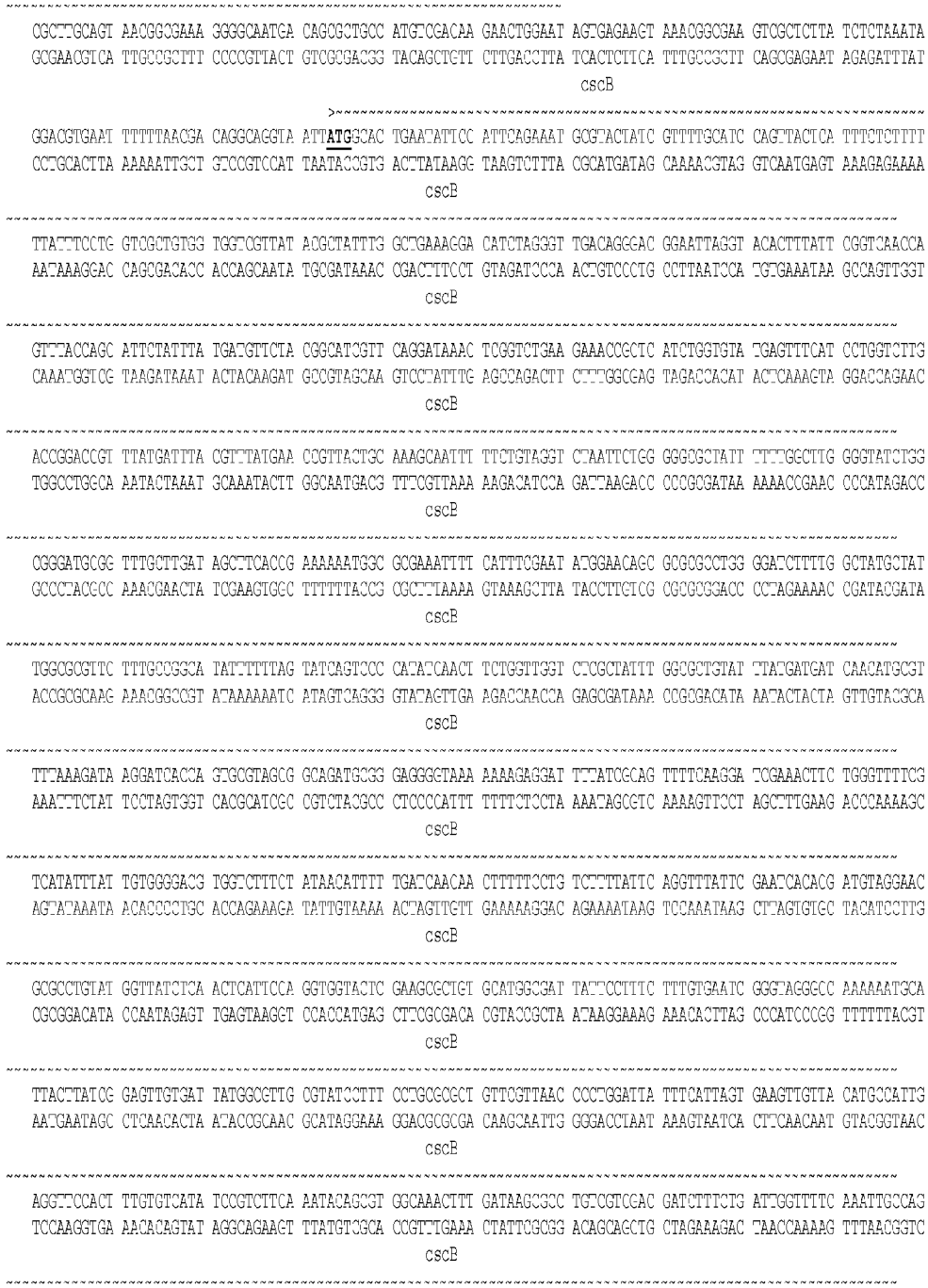
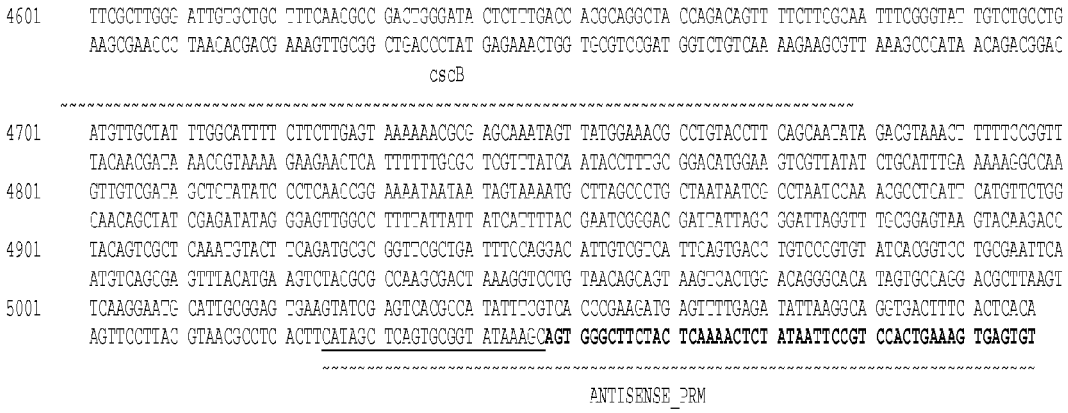


FIG. 55 (cont'd)


4601
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AAGCGAACCC TAACACGACG AAAGTTGCGG CTGACCCTAT GAGAAACTGG TGCGTCCGAT GGTCTGTCAA AAGAAGCGTT AAAGCCCATA ACAGACGGAC
cscB
4701
ATGTTGCTAT TTGGCATTTT CTTCTTGAGT AAAAAACGCG AGCAAATAGT TATGGAAACG CCTGTACCTT CAGCAATATA GACGTAAACT TTTTCCGGTT
TACAACGATA AACCGTAAAA GAAGAACTCA TTTTTTGCGC TCGTTTATCA ATACCTTTGC GGACATGGAA GTCGTTATAT CTGCATTTGA AAAAGGCCAA
4801
GTTGTCGATA GCTCTATATC CCTCAACCGG AAAATAATAA TAGTAAAATG CTTAGCCCTG CTAATAATCG CCTAATCCAA ACGCCTCATT CATGTTCTGG
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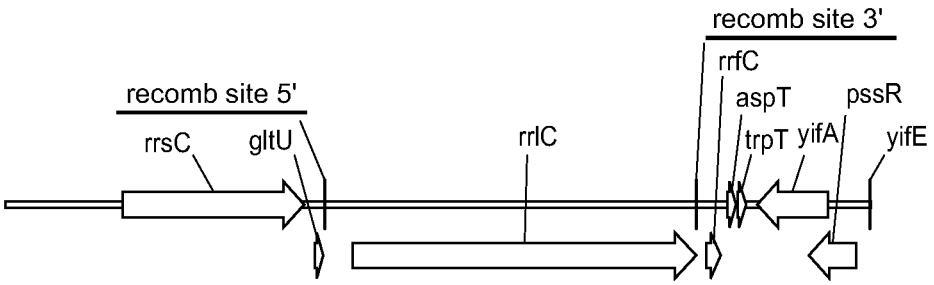
4901
TACAGTCGCT CAAATGTACT TCAGATGCGC GGTTCGCTGA TTTCCAGGAC ATTGTCGTCA TTCAGTGACC TGTCCCGTGT ATCACGGTCC TGCGAATTCA
ATGTCAGCGA GTTTACATGA AGTCTACGCG CCAAGCGACT AAAGGTCCTG TAACAGCAGT AAGTCACTGG ACAGGGCACA TAGTGCCAGG ACGCTTAAGT
5001
TCAAGGAATG CATTGCGGAG TGAAGTATCG AGTCACGCCA TATTTCGTCA CCCGAAGATG AGTTTTGAGA TATTAAGGCA GGTGACTTTC ACTCACA
AGTTCCTTAC GTAACGCCTC ACTTCATAGC TCAGTGCGGT ATAAAGCAGT GGGCTTCTAC TCAAAACTCT ATAATTCCGT CCACTGAAAG TGAGTGT
ANTISENSE_PRM

FIG. 55 (cont'd)



rrnC operon@2
7330 bp

FIG. 56

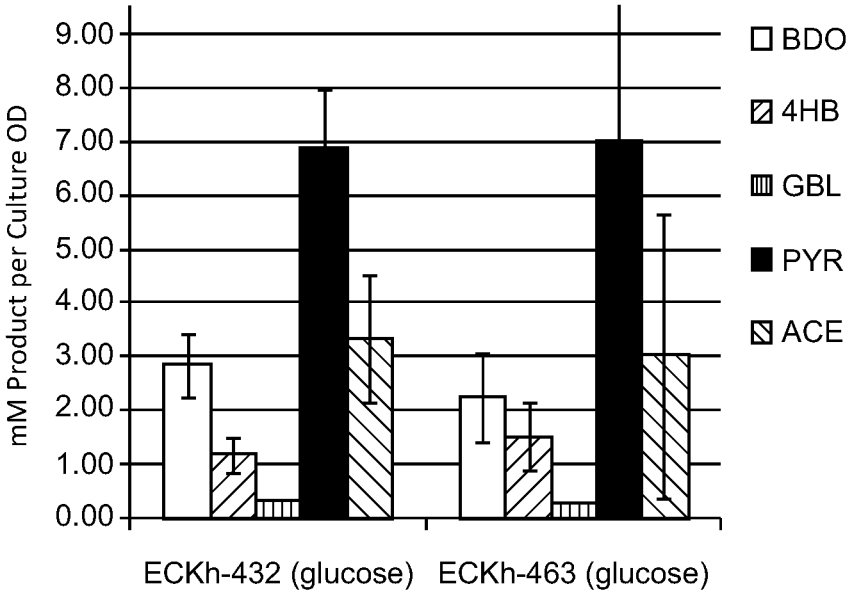


FIG. 57

MICROORGANISMS FOR THE PRODUCTION OF 1,4-BUTANEDIOL AND RELATED METHODS

This application is a continuation of application Ser. No. 12/794,700, filed Jun. 4, 2010, now U.S. Pat. No. 8,129,169, which claims the benefit of priority of U.S. application Ser. No. 61/184,311, filed Jun. 4, 2009, the entire contents of each of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Incorporated herein by reference is the Sequence Listing being concurrently submitted via EFS-Web as an ASCII text file named 12207-177-999_SeqList.txt, created Jan. 27, 2012, and being 150,347 bytes in size.

This invention relates generally to in silico design of organisms and engineering of organisms, more particularly to organisms having 1,4-butanediol biosynthesis capability.

The compound 4-hydroxybutanoic acid (4-hydroxybutanoate, 4-hydroxybutyrate, 4-HB) is a 4-carbon carboxylic acid that has industrial potential as a building block for various commodity and specialty chemicals. In particular, 4-HB has the potential to serve as a new entry point into the 1,4-butanediol family of chemicals, which includes solvents, resins, polymer precursors, and specialty chemicals. 1,4-Butanediol (BDO) is a polymer intermediate and industrial solvent with a global market of about 3 billion lb/year. BDO is currently produced from petrochemical precursors, primarily acetylene, maleic anhydride, and propylene oxide.

For example, acetylene is reacted with 2 molecules of formaldehyde in the Reppe synthesis reaction (Kroschwitz and Grant, *Encyclopedia of Chem. Tech.*, John Wiley and Sons, Inc., New York (1999)), followed by catalytic hydrogenation to form 1,4-butanediol. It has been estimated that 90% of the acetylene produced in the U.S. is consumed for butanediol production. Alternatively, it can be formed by esterification and catalytic hydrogenation of maleic anhydride, which is derived from butane. Downstream, butanediol can be further transformed; for example, by oxidation to γ-butyrolactone, which can be further converted to pyrrolidone and N-methyl-pyrrolidone, or hydrogenolysis to tetrahydrofuran. These compounds have varied uses as polymer intermediates, solvents, and additives, and have a combined market of nearly 2 billion lb/year.

It is desirable to develop a method for production of these chemicals by alternative means that not only substitute renewable for petroleum-based feedstocks, and also use less energy- and capital-intensive processes. The Department of Energy has proposed 1,4-diacids, and particularly succinic acid, as key biologically-produced intermediates for the manufacture of the butanediol family of products (DOE Report, "Top Value-Added Chemicals from Biomass", 2004). However, succinic acid is costly to isolate and purify and requires high temperatures and pressures for catalytic reduction to butanediol.

Thus, there exists a need for alternative means for effectively producing commercial quantities of 1,4-butanediol and its chemical precursors. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF INVENTION

The invention provides non-naturally occurring microbial organisms containing a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO and further optimized for expression of BDO. The invention additionally provides methods of using such microbial organisms to produce BDO.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram showing biochemical pathways to 4-hydroxybutyrate (4-HB) and to 1,4-butanediol production. The first 5 steps are endogenous to *E. coli*, while the remainder can be expressed heterologously. Enzymes catalyzing the biosynthetic reactions are: (1) succinyl-CoA synthetase; (2) CoA-independent succinic semialdehyde dehydrogenase; (3) α-ketoglutarate dehydrogenase; (4) glutamate: succinate semialdehyde transaminase; (5) glutamate decarboxylase; (6) CoA-dependent succinic semialdehyde dehydrogenase; (7) 4-hydroxybutanoate dehydrogenase; (8) α-ketoglutarate decarboxylase; (9) 4-hydroxybutyryl CoA:acetyl-CoA transferase; (10) butyrate kinase; (11) phosphotransbutyrylase; (12) aldehyde dehydrogenase; (13) alcohol dehydrogenase.

FIG. **2** is a schematic diagram showing homoserine biosynthesis in *E. coli*.

FIG. **3** shows the production of 4-HB in glucose minimal medium using *E. coli* strains harboring plasmids expressing various combinations of 4-HB pathway genes. (a) 4-HB concentration in culture broth; (b) succinate concentration in culture broth; (c) culture OD, measured at 600 nm. Clusters of bars represent the 24 hour, 48 hour, and 72 hour (if measured) timepoints. The codes along the x-axis indicate the strain/plasmid combination used. The first index refers to the host strain: 1, MG1655 lacI^Q; 2, MG1655 ΔgabD lacI^Q; 3, MG1655 ΔgabD ΔaldA lacI^Q. The second index refers to the plasmid combination used: 1, pZE13-0004-0035 and pZA33-0036; 2, pZE13-0004-0035 and pZA33-0010n; 3, pZE13-0004-0008 and pZA33-0036; 4, pZE13-0004-0008 and pZA33-0010n; 5, Control vectors pZE13 and pZA33.

FIG. **4** shows the production of 4-HB from glucose in *E. coli* strains expressing α-ketoglutarate decarboxylase from *Mycobacterium tuberculosis*. Strains 1-3 contain pZE13-0032 and pZA33-0036. Strain 4 expresses only the empty vectors pZE13 and pZA33. Host strains are as follows: 1 and 4, MG1655 lacI^Q; 2, MG1655 ΔgabD lacI^Q; 3, MG1655 ΔgabD ΔaldA lacI^Q. The bars refer to concentration at 24 and 48 hours.

FIG. **5** shows the production of BDO from 10 mM 4-HB in recombinant *E. coli* strains. Numbered positions correspond to experiments with MG1655 lacI^Q containing pZA33-0024, expressing cat2 from *P. gingivalis*, and the following genes expressed on pZE13:1, none (control); 2, 0002; 3, 0003; 4, 0003n; 5, 0011; 6, 0013; 7, 0023; 8, 0025; 9, 0008n; 10, 0035. Gene numbers are defined in Table 6. For each position, the bars refer to aerobic, microaerobic, and anaerobic conditions, respectively. Microaerobic conditions were created by sealing the culture tubes but not evacuating them.

FIG. **6** shows the mass spectrum of 4-HB and BDO produced by MG1655 lacI^Q pZE13-0004-0035-0002 pZA33-0034-0036 grown in M9 minimal medium supplemented with 4 g/L unlabeled glucose (a, c, e, and g) uniformly labeled ^{13}C-glucose (b, d, f, and h). (a) and (b), mass 116 characteristic fragment of derivatized BDO, containing 2 carbon atoms; (c) and (d), mass 177 characteristic fragment of derivatized BDO, containing 1 carbon atom; (e) and (f), mass 117 characteristic fragment of derivatized 4-HB, containing 2 carbon atoms; (g) and (h), mass 233 characteristic fragment of derivatized 4-HB, containing 4 carbon atoms.

FIG. **7** is a schematic process flow diagram of bioprocesses for the production of γ-butyrolactone. Panel (a) illustrates fed-batch fermentation with batch separation and panel (b) illustrates fed-batch fermentation with continuous separation.

FIGS. **8**A and **8**B show exemplary 1,4-butanediol (BDO) pathways. FIG. **8**A shows BDO pathways from succinyl-CoA. FIG. **8**B shows BDO pathways from alpha-ketoglutarate.

FIGS. **9**A-**9**C show exemplary BDO pathways. FIGS. **9**A and **9**B show pathways from 4-aminobutyrate. FIG. **9**C shows a pathway from acetoacetyl-CoA to 4-aminobutyrate.

FIG. **10** shows exemplary BDO pathways from alpha-ketoglutarate.

FIG. **11** shows exemplary BDO pathways from glutamate.

FIG. **12** shows exemplary BDO pathways from acetoacetyl-CoA.

FIG. **13** shows exemplary BDO pathways from homoserine.

FIG. **14** shows the nucleotide and amino acid sequences of *E. coli* succinyl-CoA synthetase. FIG. **14**A shows the nucleotide sequence (SEQ ID NO:45) of the *E. coli* sucCD operon. FIGS. **14**B (SEQ ID NO:46) and **14**C (SEQ ID NO:47) show the amino acid sequences of the succinyl-CoA synthetase subunits encoded by the sucCD operon.

FIG. **15** shows the nucleotide and amino acid sequences of *Mycobacterium bovis* alpha-ketoglutarate decarboxylase. FIG. **15**A shows the nucleotide sequence (SEQ ID NO:48) of *Mycobacterium bovis* sucA gene. FIG. **15**B shows the amino acid sequence (SEQ ID NO:49) of *M. bovis* alpha-ketoglutarate decarboxylase.

FIG. **16** shows biosynthesis in *E. coli* of 4-hydroxybutyrate from glucose in minimal medium via alpha-ketoglutarate under anaerobic (microaerobic) conditions. The host strain is ECKh-401. The experiments are labeled based on the upstream pathway genes present on the plasmid pZA33 as follows: 1) 4hbd-sucA; 2) sucCD-sucD-4-hbd; 3) sucCD-sucD-4-hbd-sucA.

FIG. **17** shows biosynthesis in *E. coli* of 4-hydroxybutyrate from glucose in minimal medium via succinate and alpha-ketoglutarate. The host strain is wild-type MG1655. The experiments are labeled based on the genes present on the plasmids pZE13 and pZA33 as follows: 1) empty control vectors 2) empty pZE13, pZA33-4-hbd; 3) pZE13-sucA, pZA33-4-hbd.

FIG. **18** A shows the nucleotide sequence (SEQ ID NO:50) of CoA-dependent succinate semialdehyde dehydrogenase (sucD) from *Porphyromonas gingivalis*, and FIG. **18**B shows the encoded amino acid sequence (SEQ ID NO:51).

FIG. **19**A shows the nucleotide sequence (SEQ ID NO:52) of 4-hydroxybutyrate dehydrogenase (4-hbc-1) from *Porphyromonas gingivalis*, and FIG. **19**B shows the encoded amino acid sequence (SEQ ID NO:53).

FIG. **20**A shows the nucleotide sequence (SEQ ID NO:54) of 4-hydroxybutyrate CoA transferase (cat2) from *Porphyromonas gingivalis*, and FIG. **20**B shows the encoded amino acid sequence (SEQ ID NO:55).

FIG. **21**A shows the nucleotide sequence (SEQ ID NO:56) of phosphotransbutyrylase (ptb) from *Clostridium acetobutylicum*, and FIG. **21**B shows the encoded amino acid sequence (SEQ ID NO:57).

FIG. **22**A shows the nucleotide sequence (SEQ ID NO:58) of butyrate kinase (buk1) from *Clostridium acetobutylicum*, and FIG. **22**B shows the encoded amino acid sequence (SEQ ID NO:59).

FIG. **23** shows alternative nucleotide sequences for *C. acetobutylicum* 020 (phosphtransbutyrylase) with altered codons for more prevalent *E. coli* codons relative to the *C. acetobutylicum* native sequence. FIGS. **23**A-**23**D (020A-020D, SEQ ID NOS:60-63, respectively) contain sequences with increasing numbers of rare *E. coli* codons replaced by more prevalent codons (A<B<C<D).

FIG. **24** shows alternative nucleotide sequences for *C. acetobuytlicum* 021 (butyrate kinase) with altered codons for more prevalent *E. coli* codons relative to the *C. acetobutylicum* native sequence. FIGS. **24**A-**24**D (021A-021B, SEQ ID NOS:64-67, respectively) contain sequences with increasing numbers of rare *E. coli* codons replaced by more prevalent codons (A<B<C<D).

FIG. **25** shows improved expression of butyrate kinase (BK) and phosphotransbutyrylase (PTB) with optimized codons for expression in *E. coli*. FIG. **25**A shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained for proteins with Coomassie blue; lane 1, control vector with no insert; lane 2, expression of *C. acetobutylicum* native sequences in *E. coli*; lane 3, expression of 020B-021B codon optimized PTB-BK; lane 4, expression of 020C-021C codon optimized PTB-BK. The positions of BK and PTB are shown. FIG. **25**B shows the BK and PTB activities of native *C. acetobutylicum* sequence (2021n) compared to codon optimized 020B-021B (2021B) and 020C-021C (2021C).

FIG. **26** shows production of BDO and gamma-butyrolactone (GBL) in various strains expressing BDO producing enzymes: Cat2 (034); 2021n; 2021B; 2021C.

FIG. **27**A shows the nucleotide sequence (SEQ ID NO:68) of the native *Clostridium beijerinckii* ald gene (025n), and FIG. **27**B shows the encoded amino acid sequence (SEQ ID NO:69).

FIGS. **28**A-**28**D show alternative gene sequences for the *Clostridium beijerinckii* ald gene (025A-025D, SEQ ID NOS:70-73, respectively), in which increasing numbers of rare codons are replaced by more prevalent codons (A<B<C<D).

FIG. **29** shows expression of native *C. beijerinckii* ald gene and codon optimized variants; no ins (control with no insert), 025n, 025A, 025B, 025C, 025D.

FIG. **30** shows BDO or BDO and ethanol production in various strains. FIG. **30**A shows BDO production in strains containing the native *C. beijerinckii* ald gene (025n) or variants with optimized codons for expression in *E. coli* (025A-025D). FIG. **30**B shows production of ethanol and BDO in strains expressing the *C. acetobutylicum* AdhE2 enzyme (002C) compared to the codon optimized variant 025B. The third set shows expression of *P. gingivalis* sucD (035). In all cases, *P. gingivalis* Cat2 (034) is also expressed.

FIG. **31**A shows the nucleotide sequence (SEQ ID NO:74) of the adh1 gene from *Geobacillus thermoglucosidasius*, and FIG. **31**B shows the encoded amino acid sequence (SEQ ID NO:75).

FIG. **32**A shows the expression of the *Geobacillus thermoglucosidasius* adh1 gene in *E. coli*. Either whole cell lysates or supernatants were analyzed by SDS-PAGE and stained with Coomassie blue for plasmid with no insert, plasmid with 083 (*Geotrichum capitatum* N-benzyl-3-pyrrolidinol dehydrogenase) and plasmid with 084 (*Geobacillus thermoglucosidasius* adh1) inserts. FIG. **32**B shows the activity of 084 with butyraldehyde (diamonds) or 4-hydroxybutyraldehyde (squares) as substrates.

FIG. **33** shows the production of BDO in various strains: plasmid with no insert; 025B, 025B-026n; 025B-026A; 025B-026B; 025B-026C; 025B-050; 025B-052; 025B-053;

025B-055; 025B-057; 025B-058; 025B-071; 025B-083; 025B-084; PTSlacO-025B; PTSlacO-025B-026n.

FIG. **34** shows a plasmid map for the vector pRE119-V2.

FIG. **35** shows the sequence (SEQ ID NO:76) of the ECKh-138 region encompassing the aceF and lpdA genes. The *K. pneumonia* lpdA gene is underlined, and the codon changed in the Glu354Lys mutant shaded.

FIG. **36** shows the protein sequence comparison of the native *E. coli* lpdA (SEQ ID NO:77) and the mutant *K. pneumonia* lpdA (SEQ ID NO:78).

FIG. **37** shows 4-hydroxybutyrate (left bars) and BDO (right bars) production in the strains AB3, MG1655 ΔldhA and ECKh-138. All strains expressed *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd on the medium copy plasmid pZA33, and *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2 on the high copy plasmid pZE13.

FIG. **38** shows the nucleotide sequence (SEQ ID NO:79) of the 5' end of the aceE gene fused to the pflB-p6 promoter and ribosome binding site (RBS). The 5' italicized sequence shows the start of the aroP gene, which is transcribed in the opposite direction from the pdh operon. The 3' italicized sequence shows the start of the aceE gene. In upper case: pflB RBS. Underlined: FNR binding site. In bold: pflB-p6 promoter sequence.

FIG. **39** shows the nucleotide sequence (SEQ ID NO:80) in the aceF-lpdA region in the strain ECKh-456.

FIG. **40** shows the production of 4-hydroxybutyrate, BDO and pyruvate (left to right bars, respectively) for each of strains ECKh-439, ECKh-455 and ECKh-456.

FIG. **41**A shows a schematic of the recombination sites for deletion of the mdh gene. FIG. **41**B shows the sequence (nucleotide sequence, SEQ ID NO:81, and coded amino acid sequence, SEQ ID NO:82) of the PCR product of the amplification of chloramphenicol resistance gene (CAT) flanked by FRT sites and homology regions from the mdh gene from the plasmid pKD3.

FIG. **42** shows the sequence (SEQ ID NO:83) of the arcA deleted region in strain ECKh-401.

FIG. **43** shows the sequence (SEQ ID NO:84) of the region encompassing a mutated gltA gene of strain ECKh-422.

FIGS. **44**A and **44**B show the citrate synthase activity of wild type gltA gene product (FIG. **44**A) and the R163L mutant (FIG. **44**B). The assay was performed in the absence (diamonds) or presence of 0.4 mM NADH (squares).

FIG. **45** shows the 4-hydroxybutyrate (left bars) and BDO (right bars) production in strains ECKh-401 and ECKh-422, both expressing genes for the complete BDO pathway on plasmids.

FIG. **46** shows central metabolic fluxes and associated 95% confidence intervals from metabolic labeling experiments. Values are molar fluxes normalized to a glucose uptake rate of 1 mmol/hr. The result indicates that carbon flux is routed through citrate synthase in the oxidative direction and that most of the carbon enters the BDO pathway rather than completing the TCA cycle.

FIGS. **47**A and **47**B show extracellular product formation for strains ECKh-138 (FIG. **47**A) and ECKh-422 (FIG. **47**B), both expressing the entire BDO pathway on plasmids. The products measured were acetate (Ace), pyruvate (Pyr), 4-hydroxybutyrate (4HB), 1,4-butanediol (BDO), ethanol (EtOH), and other products, which include gamma-butyrolactone (GBL), succinate, and lactate.

FIG. **48** shows the sequence (SEQ ID NO:85) of the region following replacement of PEP carboxylase (ppc) by *H. influenzae* phosphoenolpyruvate carboxykinase (pepck). The pepck coding region is underlined.

FIG. **49** shows growth of evolved pepCK strains grown in minimal medium containing 50 mM $NaHCO_3$.

FIG. **50** shows product formation in strain ECKh-453 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald on the plasmid pZS* 13. The products measured were 1,4-butanediol (BDO), pyruvate, 4-hydroxybutyrate (4HB), acetate, γ-butyrolactone (GBL) and ethanol.

FIG. **51** shows BDO production of two strains, ECKh-453 and ECKh-432. Both contain the plasmid pZS*13 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. The cultures were grown under microaerobic conditions, with the vessels punctured with 27 or 18 gauge needles, as indicated.

FIG. **52** shows the nucleotide sequence (SEQ ID NO:86) of the genomic DNA of strain ECKh-426 in the region of insertion of a polycistronic DNA fragment containing a promoter, sucCD gene, sucD gene, 4hbd gene and a terminator sequence.

FIG. **53** shows the nucleotide sequence (SEQ ID NO:87) of the chromosomal region of strain ECKh-432 in the region of insertion of a polycistronic sequence containing a promoter, sucA gene, *C. kluyveri* 4hbd gene and a terminator sequence.

FIG. **54** shows BDO synthesis from glucose in minimal medium in the ECKh-432 strain having upstream BDO pathway encoding genes integrated into the chromosome and containing a plasmid harboring downstream BDO pathway genes.

FIG. **55** shows a PCR product (SEQ ID NO:88) containing the non-phosphotransferase (non-PTS) sucrose utilization genes flanked by regions of homology to the rrnC region.

FIG. **56** shows a schematic diagram of the integrations site in the rrnC operon.

FIG. **57** shows average product concentration, normalized to culture OD600, after 48 hours of growth of strain ECKh-432 grown on glucose and strain ECKh-463 grown on sucrose. Both contain the plasmid pZS*13 expressing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. The data is for 6 replicate cultures of each strain. The products measured were 1,4-butanediol (BDO), 4-hydroxybutyrate (4HB), γ-butyrolactone (GBL), pyruvate (PYR) and acetate (ACE) (left to right bars, respectively).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the design and production of cells and organisms having biosynthetic production capabilities for 4-hydroxybutanoic acid (4-HB), γ-butyrolactone and 1,4-butanediol (BDO). The invention, in particular, relates to the design of microbial organisms capable of producing BDO by introducing one or more nucleic acids encoding a BDO pathway enzyme.

In one embodiment, the invention utilizes in silico stoichiometric models of *Escherichia coli* metabolism that identify metabolic designs for biosynthetic production of 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO). The results described herein indicate that metabolic pathways can be designed and recombinantly engineered to achieve the biosynthesis of 4-HB and downstream products such as 1,4-butanediol in *Escherichia coli* and other cells or organisms. Biosynthetic production of 4-HB, for example, for the in silico designs can be confirmed by construction of strains having the designed metabolic genotype. These metabolically engineered cells or organisms also can be

subjected to adaptive evolution to further augment 4-HB biosynthesis, including under conditions approaching theoretical maximum growth.

In certain embodiments, the 4-HB biosynthesis characteristics of the designed strains make them genetically stable and particularly useful in continuous bioprocesses. Separate strain design strategies were identified with incorporation of different non-native or heterologous reaction capabilities into *E. coli* or other host organisms leading to 4-HB and 1,4-butanediol producing metabolic pathways from either CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase and CoA-dependent succinic semialdehyde dehydrogenase, or glutamate: succinic semialdehyde transaminase. In silico metabolic designs were identified that resulted in the biosynthesis of 4-HB in both *E. coli* and yeast species from each of these metabolic pathways. The 1,4-butanediol intermediate γ-butyrolactone can be generated in culture by spontaneous cyclization under conditions at pH<7.5, particularly under acidic conditions, such as below pH 5.5, for example, pH<7, pH<6.5, pH<6, and particularly at pH<5.5 or lower.

Strains identified via the computational component of the platform can be put into actual production by genetically engineering any of the predicted metabolic alterations which lead to the biosynthetic production of 4-HB, 1,4-butanediol or other intermediate and/or downstream products. In yet a further embodiment, strains exhibiting biosynthetic production of these compounds can be further subjected to adaptive evolution to further augment product biosynthesis. The levels of product biosynthesis yield following adaptive evolution also can be predicted by the computational component of the system.

In other specific embodiments, microbial organisms were constructed to express a 4-HB biosynthetic pathway encoding the enzymatic steps from succinate to 4-HB and to 4-HB-CoA. Co-expression of succinate coenzyme A transferase, CoA-dependent succinic semialdehyde dehydrogenase, NAD-dependent 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyrate coenzyme A transferase in a host microbial organism resulted in significant production of 4-HB compared to host microbial organisms lacking a 4-HB biosynthetic pathway. In a further specific embodiment, 4-HB-producing microbial organisms were generated that utilized α-ketoglutarate as a substrate by introducing nucleic acids encoding α-ketoglutarate decarboxylase and NAD-dependent 4-hydroxybutyrate dehydrogenase.

In another specific embodiment, microbial organisms containing a 1,4-butanediol (BDO) biosynthetic pathway were constructed that biosynthesized BDO when cultured in the presence of 4-HB. The BDO biosynthetic pathway consisted of a nucleic acid encoding either a multifunctional aldehyde/alcohol dehydrogenase or nucleic acids encoding an aldehyde dehydrogenawse and an alcohol dehydrogenase. To support growth on 4-HB substrates, these BDO-producing microbial organisms also expressed 4-hydroxybutyrate CoA transferase or 4-butyrate kinase in conjunction with phosphotranshydroxybutyrlase. In yet a further specific embodiment, microbial organisms were generated that synthesized BDO through exogenous expression of nucleic acids encoding a functional 4-HB biosynthetic pathway and a functional BDO biosynthetic pathway. The 4-HB biosynthetic pathway consisted of succinate coenzyme A transferase, CoA-dependent succinic semialdehyde dehydrogenase, NAD-dependent 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyrate coenzyme A transferase. The BDO pathway consisted of a multifunctional aldehyde/alcohol dehydrogenase. Further described herein are additional pathways for production of BDO (see FIGS. **8-13**).

As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modification include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes or proteins within a biosynthetic pathway for a BDO family of compounds.

A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms having genetic modifications to nucleic acids encoding metabolic polypeptides or, functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

As used herein, the terms "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

As used herein, the term "4-hydroxybutanoic acid" is intended to mean a 4-hydroxy derivative of butyric acid having the chemical formula $C_4H_8O_3$ and a molecular mass of 104.11 g/mol (126.09 g/mol for its sodium salt). The chemical compound 4-hydroxybutanoic acid also is known in the art as 4-HB, 4-hydroxybutyrate, gamma-hydroxybutyric acid or GHB. The term as it is used herein is intended to include any of the compound's various salt forms and include, for example, 4-hydroxybutanoate and 4-hydroxybutyrate. Specific examples of salt forms for 4-HB include sodium 4-HB and potassium 4-HB. Therefore, the terms 4-hydroxybutanoic acid, 4-HB, 4-hydroxybutyrate, 4-hy-

droxybutanoate, gamma-hydroxybutyric acid and GHB as well as other art recognized names are used synonymously herein.

As used herein, the term "monomeric" when used in reference to 4-HB is intended to mean 4-HB in a non-polymeric or underivatized form. Specific examples of polymeric 4-HB include poly-4-hydroxybutanoic acid and copolymers of, for example, 4-HB and 3-HB. A specific example of a derivatized form of 4-HB is 4-HB-CoA. Other polymeric 4-HB forms and other derivatized forms of 4-HB also are known in the art.

As used herein, the term "γ-butyrolactone" is intended to mean a lactone having the chemical formula $C_4H_6O_2$ and a molecular mass of 86.089 g/mol. The chemical compound γ-butyrolactone also is know in the art as GBL, butyrolactone, 1,4-lactone, 4-butyrolactone, 4-hydroxybutyric acid lactone, and gamma-hydroxybutyric acid lactone. The term as it is used herein is intended to include any of the compound's various salt forms.

As used herein, the term "1,4-butanediol" is intended to mean an alcohol derivative of the alkane butane, carrying two hydroxyl groups which has the chemical formula $C_4H_{10}O_2$ and a molecular mass of 90.12 g/mol. The chemical compound 1,4-butanediol also is known in the art as BDO and is a chemical intermediate or precursor for a family of compounds referred to herein as BDO family of compounds.

As used herein, the term "tetrahydrofuran" is intended to mean a heterocyclic organic compound corresponding to the fully hydrogenated analog of the aromatic compound furan which has the chemical formula C_4H_8O and a molecular mass of 72.11 g/mol. The chemical compound tetrahydrofuran also is known in the art as THF, tetrahydrofuran, 1,4-epoxybutane, butylene oxide, cyclotetramethylene oxide, oxacyclopentane, diethylene oxide, oxolane, furanidine, hydrofuran, tetra-methylene oxide. The term as it is used herein is intended to include any of the compound's various salt forms.

As used herein, the term "CoA" or "coenzyme A" is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation, pyruvate oxidation and in other acetylation.

As used herein, the term "substantially anaerobic" when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

The non-naturally occurring microbal organisms of the invention can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein are described with reference to a suitable source organism such as *E. coli*, yeast, or other organisms disclosed herein and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes encoding enzymes for their corresponding metabolic reactions. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less that 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the growth-coupled production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and *Drosophila* DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog to either or both of the exonuclease or the polymerase from the second species and vice versa.

In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved

from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having 4-HB, GBL and/or BDO biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes.

Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan. 5, 1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sep. 16, 1998) and the following parameters: Match: 1; mismatch: −2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

Disclosed herein are non-naturally occurring microbial biocatalyst or microbial organisms including a microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway that includes at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate: succinic semialdehyde transaminase, alpha-ketoglutarate decarboxylase, or glutamate decarboxylase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce monomeric 4-hydroxybutanoic acid (4-HB). 4-hydroxybutanoate dehydrogenase is also referred to as 4-hydroxybutyrate dehydrogenase or 4-HB dehydrogenase. Succinyl-CoA synthetase is also referred to as succinyl-CoA synthase or succinyl-CoA ligase.

Also disclosed herein is a non-naturally occurring microbial biocatalyst or microbial organism including a microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway having at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, or α-ketoglutarate decarboxylase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce monomeric 4-hydroxybutanoic acid (4-HB).

The non-naturally occurring microbial biocatalysts or microbial organisms can include microbial organisms that employ combinations of metabolic reactions for biosynthetically producing the compounds of the invention. The biosynthesized compounds can be produced intracellularly and/or secreted into the culture medium. Exemplary compounds produced by the non-naturally occurring microorganisms include, for example, 4-hydroxybutanoic acid, 1,4-butanediol and γ-butyrolactone.

In one embodiment, a non-naturally occurring microbial organism is engineered to produce 4-HB. This compound is one useful entry point into the 1,4-butanediol family of compounds. The biochemical reactions for formation of 4-HB from succinate, from succinate through succinyl-CoA or from α-ketoglutarate are shown in steps 1-8 of FIG. **1**.

It is understood that any combination of appropriate enzymes of a BDO pathway can be used so long as conversion from a starting component to the BDO product is achieved. Thus, it is understood that any of the metabolic pathways disclosed herein can be utilized and that it is well understood to those skilled in the art how to select appropriate enzymes to achieve a desired pathway, as disclosed herein.

In another embodiment, disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Example VII Table 17). The BDO pathway further can comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

It is understood by those skilled in the art that various combinations of the pathways can be utilized, as disclosed herein. For example, in a non-naturally occurring microbial organism, the nucleic acids can encode 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA oxidoreductase (deaminating) or 4-aminobutyryl-CoA transaminase; and 4-hydroxybutyryl-CoA dehydrogenase. Other exemplary combinations are specifically describe below and further can be found in FIGS. **8-13**. For example, the BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Example VII and Table 18), and can further comprise 1,4-butanediol dehydrogenase. For example, the exogenous nucleic acids can encode 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA reductase (alcohol forming); and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase. In addition, the nucleic acids can encode. 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, or 4-aminobutyrate-CoA ligase; 4-aminobutyryl-CoA reductase; 4-aminobutan-1-ol dehydrogenase; and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase.

Also disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 4-aminobutyrate kinase, 4-aminobutyraldehyde dehydrogenase (phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-oloxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobutanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Example VII and Table 19). For example, the exogenous nucleic acids can encode 4-aminobutyrate kinase; 4-aminobutyraldehyde dehydrogenase (phosphorylating); 4-aminobutan-1-ol dehydrogenase; and 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase. Alternatively, the exogenous nucleic acids can encode 4-aminobutyrate kinase; [(4-aminobutanolyl)oxy] phosphonic acid oxidoreductase (deaminating) or [(4-aminobutanolyl)oxy]phosphonic acid transaminase; 4-hydroxybutyryl-phosphate dehydrogenase; and 4-hydroxybutyraldehyde dehydrogenase (phosphorylating).

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (see Example VIII and Table 20). The BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase. For example, the exogenous nucleic acids can encode alpha-ketoglutarate 5-kinase; 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating); 2,5-dioxopentanoic acid reductase; and 5-hydroxy-2-oxopentanoic acid decarboxylase. Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate 5-kinase; 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating); 2,5-dioxopentanoic acid reductase; and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase; and 5-hydroxy-2-oxopentanoic acid decarboxylase. In another embodiment, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase (alcohol forming); and 5-hydroxy-2-oxopentanoic acid decarboxylase. In yet another embodiment, the exogenous nucleic acids can encode alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, or alpha-ketoglutaryl-CoA ligase; alpha-ketoglutaryl-CoA reductase (alcohol forming); and 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation).

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (see Example

IX and Table 21). For example, the exogenous nucleic acids can encode glutamate CoA transferase, glutamyl-CoA hydrolase, or glutamyl-CoA ligase; glutamyl-CoA reductase; glutamate-5-semialdehyde reductase; 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). Alternatively, the exogenous nucleic acids can encode glutamate 5-kinase; glutamate-5-semialdehyde dehydrogenase (phosphorylating); glutamate-5-semialdehyde reductase; 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). In still another embodiment, the exogenous nucleic acids can encode glutamate CoA transferase, glutamyl-CoA hydrolase, or glutamyl-CoA ligase; glutamyl-CoA reductase (alcohol forming); 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation). In yet another embodiment, the exogenous nucleic acids can encode glutamate 5-kinase; glutamate-5-semialdehyde dehydrogenase (phosphorylating); 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) or 2-amino-5-hydroxypentanoic acid transaminase; and 5-hydroxy-2-oxopentanoic acid decarboxylase or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation).

Also disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA A-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Example X and Table 22). For example, the exogenous nucleic acids can encode 3-hydroxybutyryl-CoA dehydrogenase; 3-hydroxybutyryl-CoA dehydratase; vinylacetyl-CoA A-isomerase; and 4-hydroxybutyryl-CoA dehydratase.

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Example XI and Table 23). For example, the exogenous nucleic acids can encode homoserine deaminase; 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase; 4-hydroxybut-2-enoyl-CoA reductase. Alternatively, the exogenous nucleic acids can encode homoserine CoA transferase, homoserine-CoA hydrolase, or homoserine-CoA ligase; homoserine-CoA deaminase; and 4-hydroxybut-2-enoyl-CoA reductase. In a further embodiment, the exogenous nucleic acids can encode homoserine deaminase; 4-hydroxybut-2-enoate reductase; and 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, or 4-hydroxybutyryl-CoA ligase. Alternatively, the exogenous nucleic acids can encode homoserine CoA transferase, homoserine-CoA hydrolase, or homoserine-CoA ligase; homoserine-CoA deaminase; and 4-hydroxybut-2-enoyl-CoA reductase.

Further disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BOD, the BDO pathway comprising succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating) (see Table 15). Such a BDO pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

Additionally disclosed herein is a non-naturally occurring microbial organism, comprising a microbial organism having a BDO pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, the BDO pathway comprising glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating)(see Table 16). Such a BDO pathway can further comprise alpha-ketoglutarate decarboxylase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

The pathways described above are merely exemplary. One skilled in the art can readily select appropriate pathways from those disclosed herein to obtain a suitable BDO pathway or other metabolic pathway, as desired.

The invention provides genetically modified organisms that allow improved production of a desired product such as BDO by increasing the product or decreasing undesirable byproducts. As disclosed herein, the invention provides a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In one embodiment, the microbial organism is genetically modified to express exogenous succinyl-CoA synthetase (see Example XII). For example, the succinyl-CoA synthetase can be encoded by an *Escherichia coli* sucCD genes.

In another embodiment, the microbial organism is genetically modified to express exogenous alpha-ketoglutarate decarboxylase (see Example XIII). For example, the alpha-ketoglutarate decarboxylase can be encoded by the *Mycobacterium bovis* sucA gene. In still another embodiment, the microbial organism is genetically modified to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase (see Example XIII). For example, the succinate semialdehyde dehydrogenase (CoA-dependent), 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA/acetyl-CoA transferase can be encoded by *Porphyromonas gingivalis* W83 genes. In an additional embodiment, the microbial organism is genetically modified to express exogenous butyrate kinase and phosphotransbutyrylase (see Example XIII). For example, the butyrate

kinase and phosphotransbutyrylase can be encoded by *Clostridium acetobutilicum* buk1 and ptb genes.

In yet another embodiment, the microbial organism is genetically modified to express exogenous 4-hydroxybutyryl-CoA reductase (see Example XIII). For example, the 4-hydroxybutyryl-CoA reductase can be encoded by *Clostridium beijerinckii* ald gene. Additionally, in an embodiment of the invention, the microbial organism is genetically modified to express exogenous 4-hydroxybutanal reductase (see Example XIII). For example, the 4-hydroxybutanal reductase can be encoded by *Geobacillus thermoglucosidasius* adh1 gene. In another embodiment, the microbial organism is genetically modified to express exogenous pyruvate dehydrogenase subunits (see Example XIV). For example, the exogenous pyruvate dehydrogenase can be NADH insensitive. The pyruvate dehydrogenase subunit can be encoded by the *Klebsiella pneumonia* lpdA gene. In a particular embodiment, the pyruvate dehydrogenase subunit genes of the microbial organism can be under the control of a pyruvate formate lyase promoter.

In still another embodiment, the microbial organism is genetically modified to disrupt a gene encoding an aerobic respiratory control regulatory system (see Example XV). For example, the disruption can be of the arcA gene. Such an organism can further comprise disruption of a gene encoding malate dehydrogenase. In a further embodiment, the microbial organism is genetically modified to express an exogenous NADH insensitive citrate synthase (see Example XV). For example, the NADH insensitive citrate synthase can be encoded by gltA, such as an R163L mutant of gltA. In still another embodiment, the microbial organism is genetically modified to express exogenous phosphoenolpyruvate carboxykinase (see Example XVI). For example, the phosphoenolpyruvate carboxykinase can be encoded by an *Haemophilus influenza* phosphoenolpyruvate carboxykinase gene.

It is understood that any of a number of genetic modifications, as disclosed herein, can be used alone or in various combinations of one or more of the genetic modifications disclosed herein to increase the production of BDO in a BDO producing microbial organism. In a particular embodiment, the microbial organism can be genetically modified to incorporate any and up to all of the genetic modifications that lead to increased production of BDO. In a particular embodiment, the microbial organism containing a BDO pathway can be genetically modified to express exogenous succinyl-CoA synthetase; to express exogenous alpha-ketoglutarate decarboxylase; to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase; to express exogenous butyrate kinase and phosphotransbutyrylase; to express exogenous 4-hydroxybutyryl-CoA reductase; and to express exogenous 4-hydroxybutanal reductase; to express exogenous pyruvate dehydrogenase; to disrupt a gene encoding an aerobic respiratory control regulatory system; to express an exogenous NADH insensitive citrate synthase; and to express exogenous phosphoenolpyruvate carboxykinase. Such strains for improved production are described in Examples XII-XIX. It is thus understood that, in addition to the modifications described above, such strains can additionally include other modifications disclosed herein. Such modifications include, but are not limited to, deletion of endogenous lactate dehydrogenase (ldhA), alcohol dehydrogenase (adhE), and/or pyruvate formate lyase (pflB)(see Examples XII-XIX and Table 28).

Additionally provided is a microbial organism in which one or more genes encoding the exogenously expressed enzymes are integrated into the fimD locus of the host organism (see Example XVII). For example, one or more genes encoding a BDO pathway enzyme can be integrated into the fimD locus for increased production of BDO. Further provided is a microbial organism expressing a non-phosphotransferase sucrose uptake system that increases production of BDO.

Although the genetically modified microbial organisms disclosed herein are exemplified with microbial organisms containing particular BDO pathway enzymes, it is understood that such modifications can be incorporated into any microbial organism having a BDO pathway suitable for enhanced production in the presence of the genetic modifications. The microbial organisms of the invention can thus have any of the BDO pathways disclosed herein. For example, the BDO pathway can comprise 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-butyrate kinase, phosphotransbutyrylase, alpha-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase (see FIG. **1**). Alternatively, the BDO pathway can comprise 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Table 17). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase

Additionally, the BDO pathway can comprise 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Table 18). Also, the BDO pathway can comprise 4-aminobutyrate kinase, 4-aminobutyraldehyde dehydrogenase (phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-oloxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobutanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Table 19). Such a pathway can further comprise 1,4-butanediol dehydrogenase.

The BDO pathway can also comprise alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Table 20). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase. Additionally, the BDO pathway can comprise glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-

hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (see Table 21). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

Additionally, the BDO pathway can comprise 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA A-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Table 22). Also, the BDO pathway can comprise homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Table 23). Such a BDO pathway can further comprise 4-hydroxybutyryl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA reductase, or 1,4-butanediol dehydrogenase.

The BDO pathway can additionally comprise succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybutanal dehydrogenase (phosphorylating) (see Table 15). Such a pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase. Also, the BDO pathway can comprise glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybutanal dehydrogenase (phosphorylating) (see Table 16). Such a BDO pathway can further comprise alpha-ketoglutarate decarboxylase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or genes encoding an enzyme associated with or catalyzing the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction and that reference to any of these metabolic constitutes also references the gene or genes encoding the enzymes that catalyze the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes as well as the reactants and products of the reaction.

The production of 4-HB via biosynthetic modes using the microbial organisms of the invention is particularly useful because it can produce monomeric 4-HB. The non-naturally occurring microbial organisms of the invention and their biosynthesis of 4-HB and BDO family compounds also is particularly useful because the 4-HB product can be (1) secreted; (2) can be devoid of any derivatizations such as Coenzyme A; (3) avoids thermodynamic changes during biosynthesis; (4) allows direct biosynthesis of BDO, and (5) allows for the spontaneous chemical conversion of 4-HB to γ-butyrolactone (GBL) in acidic pH medium. This latter characteristic also is particularly useful for efficient chemical synthesis or biosynthesis of BDO family compounds such as 1,4-butanediol and/or tetrahydrofuran (THF), for example.

Microbial organisms generally lack the capacity to synthesize 4-HB and therefore any of the compounds disclosed herein to be within the 1,4-butanediol family of compounds or known by those in the art to be within the 1,4-butanediol family of compounds. Moreover, organisms having all of the requisite metabolic enzymatic capabilities are not known to produce 4-HB from the enzymes described and biochemical pathways exemplified herein. Rather, with the possible exception of a few anaerobic microorganisms described further below, the microorganisms having the enzymatic capability use 4-HB as a substrate to produce, for example, succinate. In contrast, the non-naturally occurring microbial organisms of the invention can generate 4-HB or BDO as a product. As described above, the biosynthesis of 4-HB in its monomeric form is not only particularly useful in chemical synthesis of BDO family of compounds, it also allows for the further biosynthesis of BDO family compounds and avoids altogether chemical synthesis procedures.

The non-naturally occurring microbial organisms of the invention that can produce 4-HB or BDO are produced by ensuring that a host microbial organism includes functional capabilities for the complete biochemical synthesis of at least one 4-HB or BDO biosynthetic pathway of the invention. Ensuring at least one requisite 4-HB or BDO biosynthetic pathway confers 4-HB biosynthesis capability onto the host microbial organism.

Five 4-HB biosynthetic pathways are exemplified herein and shown for purposes of illustration in FIG. **1**. Additional 4-HB and BDO pathways are described in FIGS. **8-13**. One 4-HB biosynthetic pathway includes the biosynthesis of 4-HB from succinate (the succinate pathway). The enzymes participating in this 4-HB pathway include CoA-independent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase. In this pathway, CoA-independent succinic semialdehyde dehydrogenase catalyzes the reverse reaction to the arrow shown in FIG. **1**. Another 4-HB biosynthetic pathway includes the biosynthesis from succinate through succinyl-CoA (the succinyl-CoA pathway). The enzymes participating in this 4-HB pathway include succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase. Three other 4-HB biosynthetic pathways include the biosynthesis of 4-HB from α-ketoglutarate (the α-ketoglutarate pathways). Hence, a third 4-HB biosynthetic pathway is the biosynthesis of succinic semialdehyde through glutamate: succinic semialdehyde transaminase, glutamate decarboxylase and 4-hydroxybutanoate dehydrogenase. A fourth 4-HB biosynthetic pathway also includes the biosynthesis of 4-HB from α-ketoglutarate, but utilizes α-ketoglutarate decarboxylase to catalyze succinic semialdehyde synthesis. 4-hydroxybutanoate dehydrogenase catalyzes the conversion of succinic semialdehyde to 4-HB. A fifth 4-HB biosynthetic pathway includes the biosynthesis from α-ketoglutarate through succinyl-CoA and utilizes α-ketoglutarate dehydrogenase to produce succinyl-CoA, which funnels into the succinyl-CoA pathway described above. Each of these 4-HB biosynthetic pathways, their substrates, reactants and products are described further below in the Examples. As

described herein, 4-HB can further be biosynthetically converted to BDO by inclusion of appropriate enzymes to produce BDO (see Example). Thus, it is understood that a 4-HB pathway can be used with enzymes for converting 4-HB to BDO to generate a BDO pathway.

The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes participating in one or more 4-HB or BDO biosynthetic pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of a particular 4-HB or BDO biosynthetic pathway can be expressed. For example, if a chosen host is deficient in one or more enzymes in a desired biosynthetic pathway, for example, the succinate to 4-HB pathway, then expressible nucleic acids for the deficient enzyme(s), for example, both CoA-independent succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase in this example, are introduced into the host for subsequent exogenous expression. Alternatively, if the chosen host exhibits endogenous expression of some pathway enzymes, but is deficient in others, then an encoding nucleic acid is needed for the deficient enzyme(s) to achieve 4-HB or BDO biosynthesis. For example, if the chosen host exhibits endogenous CoA-independent succinic semialdehyde dehydrogenase, but is deficient in 4-hydroxybutanoate dehydrogenase, then an encoding nucleic acid is needed for this enzyme to achieve 4-HB biosynthesis. Thus, a non-naturally occurring microbial organism of the invention can be produced by introducing exogenous enzyme or protein activities to obtain a desired biosynthetic pathway or a desired biosynthetic pathway can be obtained by introducing one or more exogenous enzyme or protein activities that, together with one or more endogenous enzymes or proteins, produces a desired product such as 4-HB or BDO.

In like fashion, where 4-HB biosynthesis is selected to occur through the succinate to succinyl-CoA pathway (the succinyl-CoA pathway), encoding nucleic acids for host deficiencies in the enzymes succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase and/or 4-hydroxybutanoate dehydrogenase are to be exogenously expressed in the recipient host. Selection of 4-HB biosynthesis through the α-ketoglutarate to succinic semialdehyde pathway (the α-ketoglutarate pathway) can utilize exogenous expression for host deficiencies in one or more of the enzymes for glutamate:succinic semialdehyde transaminase, glutamate decarboxylase and/or 4-hydroxybutanoate dehydrogenase, or α-ketoglutarate decarboxylase and 4-hydroxybutanoate dehydrogenase. One skilled in the art can readily determine pathway enzymes for production of 4-HB or BDO, as disclosed herein.

Depending on the 4-HB or BDO biosynthetic pathway constituents of a selected host microbial organism, the non-naturally occurring microbial organisms of the invention will include at least one exogenously expressed 4-HB or BDO pathway-encoding nucleic acid and up to all encoding nucleic acids for one or more 4-HB or BDO biosynthetic pathways. For example, 4-HB or BDO biosynthesis can be established in a host deficient in a pathway enzyme or protein through exogenous expression of the corresponding encoding nucleic acid. In a host deficient in all enzymes or proteins of a 4-HB or BDO pathway, exogenous expression of all enzyme or proteins in the pathway can be included, although it is understood that all enzymes or proteins of a pathway can be expressed even if the host contains at least one of the pathway enzymes or proteins. For example, 4-HB biosynthesis can be established from all five pathways in a host deficient in 4-hydroxybutanoate dehydrogenase through exogenous expression of a 4-hydroxybutanoate dehydrogenase encoding nucleic acid. In contrast, 4-HB biosynthesis can be established from all five pathways in a host deficient in all eight enzymes through exogenous expression of all eight of CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate: succinic semialdehyde transaminase, glutamate decarboxylase, α-ketoglutarate decarboxylase, α-ketoglutarate dehydrogenase and 4-hydroxybutanoate dehydrogenase.

Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will, at least, parallel the 4-HB or BDO pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, five, six, seven, eight or up to all nucleic acids encoding the enzymes disclosed herein constituting one or more 4-HB or BDO biosynthetic pathways. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize 4-HB or BDO biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the synthesis of one or more of the 4-HB pathway precursors such as succinate, succinyl-CoA, α-ketoglutarate, 4-aminobutyrate, glutamate, acetoacetyl-CoA, and/or homoserine.

Generally, a host microbial organism is selected such that it produces the precursor of a 4-HB or BDO pathway, either as a naturally produced molecule or as an engineered product that either provides de novo production of a desired precursor or increased production of a precursor naturally produced by the host microbial organism. For example, succinyl-CoA, α-ketoglutarate, 4-aminobutyrate, glutamate, acetoacetyl-CoA, and homoserine are produced naturally in a host organism such as *E. coli*. A host organism can be engineered to increase production of a precursor, as disclosed herein. In addition, a microbial organism that has been engineered to produce a desired precursor can be used as a host organism and further engineered to express enzymes or proteins of a 4-HB or BDO pathway.

In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize 4-HB or BDO. In this specific embodiment it can be useful to increase the synthesis or accumulation of a 4-HB or BDO pathway product to, for example, drive 4-HB or BDO pathway reactions toward 4-HB or BDO production. Increased synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the 4-HB or BDO pathway enzymes disclosed herein. Over expression of the 4-HB or BDO pathway enzyme or enzymes can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of the heterologous gene or genes. Therefore, naturally occurring organisms can be readily generated to be non-naturally 4-HB or BDO producing microbial organisms of the invention through overexpression of one, two, three, four, five, six and so forth up to all nucleic acids encoding 4-HB or BDO biosynthetic pathway enzymes. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the 4-HB or BDO biosynthetic pathway.

In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regulatory element for an exogenous gene introduced into a non-naturally occurring microbial organism (see Examples).

"Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

Sources of encoding nucleic acids for a 4-HB or BDO pathway enzyme can include, for example, any species where the encoded gene product is capable of catalyzing the referenced reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. Exemplary species for such sources include, for example, *Escherichia coli, Saccharomyces cerevisiae, Saccharomyces kluyveri, Clostridium kluyveri, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum, Clostridium perfringens, Clostridium difficile, Clostridium botulinum, Clostridium tyrobutyricum, Clostridium tetanomorphum, Clostridium tetani, Clostridium propionicum, Clostridium aminobutyricum, Clostridium subterminale, Clostridium sticklandii, Ralstonia eutropha, Mycobacterium bovis, Mycobacterium tuberculosis, Porphyromonas gingivalis, Arabidopsis thaliana, Thermus thermophilus, Pseudomonas* species, including *Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas stutzeri, Pseudomonas fluorescens, Homo sapiens, Oryctolagus cuniculus, Rhodobacter spaeroides, Thermoanaerobacter brockii, Metallosphaera sedula, Leuconostoc mesenteroides, Chloroflexus aurantiacus, Roseiflexus castenholzii, Erythrobacter, Simmondsia chinensis, Acinetobacter* species, including *Acinetobacter calcoaceticus* and *Acinetobacter baylyi, Porphyromonas gingivalis, Sulfolobus tokodaii, Sulfolobus solfataricus, Sulfolobus acidocaldarius, Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Bacillus brevis, Bacillus pumilus, Rattus norvegicus, Klebsiella pneumonia, Klebsiella oxytoca, Euglena gracilis, Treponema denticola, Moorella thermoacetica, Thermotoga maritima, Halobacterium salinarum, Geobacillus stearothermophilus, Aeropyrum pernix, Sus scrofa, Caenorhabditis elegans, Corynebacterium glutamicum, Acidaminococcus fermentans, Lactococcus lactis, Lactobacillus plantarum, Streptococcus thermophilus, Enterobacter aerogenes, Candida, Aspergillus terreus, Pedicoccus pentosaceus, Zymomonas mobilus, Acetobacter pasteurians, Kluyveromyces lactis, Eubacterium barkeri, Bacteroides capillosus, Anaerotruncus colihominis, Natranaerobius thermophilusm, Campylobacter jejuni, Haemophilus influenzae, Serratia marcescens, Citrobacter amalonaticus, Myxococcus xanthus, Fusobacterium nuleatum, Penicillium chrysogenum* marine gamma proteobacterium, butyrate-producing bacterium, and others disclosed herein (see Examples). For example, microbial organisms having 4-HB or BDO biosynthetic production are exemplified herein with reference to *E. coli* and yeast hosts. However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite 4-HB or BDO biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling biosynthesis of 4-HB or BDO and other compounds of the invention described herein with reference to a particular organism such as *E. coli* or yeast can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

In some instances, such as when an alternative 4-HB or BDO biosynthetic pathway exists in an unrelated species, 4-HB or BDO biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual genes usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize 4-HB, such as monomeric 4-HB, or BDO.

Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *Escheri-*

chia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluorescens, and *Pseudomonas putida*. Exemplary yeasts or fungi include species selected from *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger* and *Pichia pastoris*. *E. coli* is a particularly useful host organisms since it is a well characterized microbial organism suitable for genetic engineering. Other particularly useful host organisms include yeast such as *Saccharomyces cerevisiae*.

Methods for constructing and testing the expression levels of a non-naturally occurring 4-HB- or BDO-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Md. (1999). 4-HB and GBL can be separated by, for example, HPLC using a Spherisorb 5 ODS1 column and a mobile phase of 70% 10 mM phosphate buffer (pH=7) and 30% methanol, and detected using a UV detector at 215 nm (Hennessy et al. 2004, J. Forensic Sci. 46(6):1-9). BDO is detected by gas chromatography or by HPLC and refractive index detector using an Aminex HPX-87H column and a mobile phase of 0.5 mM sulfuric acid (Gonzalez-Pajuelo et al., *Met. Eng.* 7:329-336 (2005)).

Exogenous nucleic acid sequences involved in a pathway for production of 4-HB or BDO can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. coli* or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hoffmeister et al., *J. Biol. Chem.* 280:4329-4338 (2005)). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

An expression vector or vectors can be constructed to harbor one or more 4-HB biosynthetic pathway and/or one or more BDO biosynthetic encoding nucleic acids as exemplified herein operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified herein to exogenously express at least one nucleic acid encoding a 4-HB or BDO pathway enzyme in sufficient amounts to produce 4-HB, such as monomeric 4-HB, or BDO. It is understood that the microbial organisms of the invention are cultured under conditions sufficient to produce 4-HB or BDO. Exemplary levels of expression for 4-HB enzymes in each pathway are described further below in the Examples. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of 4-HB, such as monomeric 4-HB, or BDO resulting in intracellular concentrations between about 0.1-200 mM or more, for example, 0.1-25 mM or more. Generally, the intracellular concentration of 4-HB, such as monomeric 4-HB, or BDO is between about 3-150 mM or more, particularly about 5-125 mM or more, and more particularly between about 8-100 mM, for example, about 3-20 mM, particularly between about 5-15 mM and more particularly between about 8-12 mM, including about 10 mM, 20 mM, 50 mM, 80 mM or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention. In particular embodiments, the microbial organisms of the invention, particularly strains such as those disclosed herein (see Examples XII-XIX and Table 28), can provide improved production of a desired product such as BDO by increasing the production of BDO and/or decreasing undesirable byproducts. Such production levels include, but are not limited to, those disclosed herein and including from about 1 gram to about 25 grams per liter, for example about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or even higher amounts of product per liter.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described herein and are described, for example, in U.S. patent application Ser. No. 11/891,602, filed Aug. 10, 2007. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the 4-HB or BDO producers can synthesize 4-HB or BDO at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified herein. It is understood that, even though the above description refers to intracellular concentrations, 4-HB or BDO producing microbial organisms can produce 4-HB or BDO intracellularly and/or secrete the product into the culture medium.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described herein, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

As described herein, one exemplary growth condition for achieving biosynthesis of 4-HB or BDO includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

The invention also provides a non-naturally occurring microbial biocatalyst including a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways that include at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, glutamate: succinic semialdehyde transaminase, glutamate decarboxylase, CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or alcohol dehydrogenase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce 1,4-butanediol (BDO). 4-Hydroxybutyrate:CoA transferase also is known as 4-hydroxybutyryl CoA:acetyl-CoA transferase. Additional 4-HB or BDO pathway enzymes are also disclosed herein (see Examples and FIGS. **8-13**).

The invention further provides non-naturally occurring microbial biocatalyst including a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways, the pathways include at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-butyrate kinase, phosphotransbutyrylase, α-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase, wherein the exogenous nucleic acid is expressed in sufficient amounts to produce 1,4-butanediol (BDO).

Non-naturally occurring microbial organisms also can be generated which biosynthesize BDO. As with the 4-HB producing microbial organisms of the invention, the BDO producing microbial organisms also can produce intracellularly or secret the BDO into the culture medium. Following the teachings and guidance provided previously for the construction of microbial organisms that synthesize 4-HB, additional BDO pathways can be incorporated into the 4-HB producing microbial organisms to generate organisms that also synthesize BDO and other BDO family compounds. The chemical synthesis of BDO and its downstream products are known. The non-naturally occurring microbial organisms of the invention capable of BDO biosynthesis circumvent these chemical synthesis using 4-HB as an entry point as illustrated in FIG. **1**. As described further below, the 4-HB producers also can be used to chemically convert 4-HB to GBL and then to BDO or THF, for example. Alternatively, the 4-HB producers can be further modified to include biosynthetic capabilities for conversion of 4-HB and/or GBL to BDO.

The additional BDO pathways to introduce into 4-HB producers include, for example, the exogenous expression in a host deficient background or the overexpression of one or more of the enzymes exemplified in FIG. **1** as steps 9-13. One such pathway includes, for example, the enzyme activities necessary to carryout the reactions shown as steps 9, 12 and 13 in FIG. **1**, where the aldehyde and alcohol dehydrogenases can be separate enzymes or a multifunctional enzyme having both aldehyde and alcohol dehydrogenase activity. Another such pathway includes, for example, the enzyme activities necessary to carry out the reactions shown as steps 10, 11, 12 and 13 in FIG. **1**, also where the aldehyde and alcohol dehydrogenases can be separate enzymes or a multifunctional enzyme having both aldehyde and alcohol dehydrogenase activity. Accordingly, the additional BDO pathways to introduce into 4-HB producers include, for example, the exogenous expression in a host deficient background or the overexpression of one or more of a 4-hydroxybutyrate:CoA transferase, butyrate kinase, phosphotransbutyrylase, CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or an alcohol dehydrogenase. In the absence of endogenous acyl-CoA synthetase capable of modifying 4-HB, the non-naturally occurring BDO producing microbial organisms can further include an exogenous acyl-CoA synthetase selective for 4-HB, or the combination of multiple enzymes that have as a net reaction conversion of 4-HB into 4-HB-CoA. As exemplified further below in the Examples, butyrate kinase and phosphotransbutyrylase exhibit BDO pathway activity and catalyze the conversions illustrated in FIG. **1** with a 4-HB substrate. Therefore, these enzymes also can be referred to herein as 4-hydroxybutyrate kinase and phosphotranshydroxybutyrylase respectively.

Exemplary alcohol and aldehyde dehydrogenases that can be used for these in vivo conversions from 4-HB to BDO are listed below in Table 1.

TABLE 1

Alcohol and Aldehyde Dehydrogenases for Conversion of 4-HB to BDO.

ALCOHOL DEHYDROGENASES	
ec: 1.1.1.1	alcohol dehydrogenase
ec: 1.1.1.2	alcohol dehydrogenase (NADP+)
ec: 1.1.1.4	(R,R)-butanediol dehydrogenase
ec: 1.1.1.5	acetoin dehydrogenase
ec: 1.1.1.6	glycerol dehydrogenase
ec: 1.1.1.7	propanediol-phosphate dehydrogenase

TABLE 1-continued

Alcohol and Aldehyde Dehydrogenases for Conversion of 4-HB to BDO.

ec: 1.1.1.8	glycerol-3-phosphate dehydrogenase (NAD+)
ec: 1.1.1.11	D-arabinitol 4-dehydrogenase
ec: 1.1.1.12	L-arabinitol 4-dehydrogenase
ec: 1.1.1.13	L-arabinitol 2-dehydrogenase
ec: 1.1.1.14	L-iditol 2-dehydrogenase
ec: 1.1.1.15	D-iditol 2-dehydrogenase
ec: 1.1.1.16	galactitol 2-dehydrogenase
ec: 1.1.1.17	mannitol-1-phosphate 5-dehydrogenase
ec: 1.1.1.18	inositol 2-dehydrogenase
ec: 1.1.1.21	aldehyde reductase
ec: 1.1.1.23	histidinol dehydrogenase
ec: 1.1.1.26	glyoxylate reductase
ec: 1.1.1.27	L-lactate dehydrogenase
ec: 1.1.1.28	D-lactate dehydrogenase
ec: 1.1.1.29	glycerate dehydrogenase
ec: 1.1.1.30	3-hydroxybutyrate dehydrogenase
ec: 1.1.1.31	3-hydroxyisobutyrate dehydrogenase
ec: 1.1.1.35	3-hydroxyacyl-CoA dehydrogenase
ec: 1.1.1.36	acetoacetyl-CoA reductase
ec: 1.1.1.37	malate dehydrogenase
ec: 1.1.1.38	malate dehydrogenase (oxaloacetate-decarboxylating)
ec: 1.1.1.39	malate dehydrogenase (decarboxylating)
ec: 1.1.1.40	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+)
ec: 1.1.1.41	isocitrate dehydrogenase (NAD+)
ec: 1.1.1.42	isocitrate dehydrogenase (NADP+)
ec: 1.1.1.54	allyl-alcohol dehydrogenase
ec: 1.1.1.55	lactaldehyde reductase (NADPH)
ec: 1.1.1.56	ribitol 2-dehydrogenase
ec: 1.1.1.59	3-hydroxypropionate dehydrogenase
ec: 1.1.1.60	2-hydroxy-3-oxopropionate reductase
ec: 1.1.1.61	4-hydroxybutyrate dehydrogenase
ec: 1.1.1.66	omega-hydroxydecanoate dehydrogenase
ec: 1.1.1.67	mannitol 2-dehydrogenase
ec: 1.1.1.71	alcohol dehydrogenase [NAD(P)+]
ec: 1.1.1.72	glycerol dehydrogenase (NADP+)
ec: 1.1.1.73	octanol dehydrogenase
ec: 1.1.1.75	(R)-aminopropanol dehydrogenase
ec: 1.1.1.76	(S,S)-butanediol dehydrogenase
ec: 1.1.1.77	lactaldehyde reductase
ec: 1.1.1.78	methylglyoxal reductase (NADH-dependent)
ec: 1.1.1.79	glyoxylate reductase (NADP+)
ec: 1.1.1.80	isopropanol dehydrogenase (NADP+)
ec: 1.1.1.81	hydroxypyruvate reductase
ec: 1.1.1.82	malate dehydrogenase (NADP+)
ec: 1.1.1.83	D-malate dehydrogenase (decarboxylating)
ec: 1.1.1.84	dimethylmalate dehydrogenase
ec: 1.1.1.85	3-isopropylmalate dehydrogenase
ec: 1.1.1.86	ketol-acid reductoisomerase
ec: 1.1.1.87	homoisocitrate dehydrogenase
ec: 1.1.1.88	hydroxymethylglutaryl-CoA reductase
ec: 1.1.1.90	aryl-alcohol dehydrogenase
ec: 1.1.1.91	aryl-alcohol dehydrogenase (NADP+)
ec: 1.1.1.92	oxaloglycolate reductase (decarboxylating)
ec: 1.1.1.94	glycerol-3-phosphate dehydrogenase [NAD(P)+]
ec: 1.1.1.95	phosphoglycerate dehydrogenase
ec: 1.1.1.97	3-hydroxybenzyl-alcohol dehydrogenase
ec: 1.1.1.101	acylglycerone-phosphate reductase
ec: 1.1.1.103	L-threonine 3-dehydrogenase
ec: 1.1.1.104	4-oxoproline reductase
ec: 1.1.1.105	retinol dehydrogenase
ec: 1.1.1.110	indolelactate dehydrogenase

TABLE 1-continued

Alcohol and Aldehyde Dehydrogenases for Conversion of 4-HB to BDO.

ec: 1.1.1.112	indanol dehydrogenase
ec: 1.1.1.113	L-xylose 1-dehydrogenase
ec: 1.1.1.129	L-threonate 3-dehydrogenase
ec: 1.1.1.137	ribitol-5-phosphate 2-dehydrogenase
ec: 1.1.1.138	mannitol 2-dehydrogenase (NADP+)
ec: 1.1.1.140	sorbitol-6-phosphate 2-dehydrogenase
ec: 1.1.1.142	D-pinitol dehydrogenase
ec: 1.1.1.143	sequoyitol dehydrogenase
ec: 1.1.1.144	perillyl-alcohol dehydrogenase
ec: 1.1.1.156	glycerol 2-dehydrogenase (NADP+)
ec: 1.1.1.157	3-hydroxybutyryl-CoA dehydrogenase
ec: 1.1.1.163	cyclopentanol dehydrogenase
ec: 1.1.1.164	hexadecanol dehydrogenase
ec: 1.1.1.165	2-alkyn-1-ol dehydrogenase
ec: 1.1.1.166	hydroxycyclohexanecarboxylate dehydrogenase
ec: 1.1.1.167	hydroxymalonate dehydrogenase
ec: 1.1.1.174	cyclohexane-1,2-diol dehydrogenase
ec: 1.1.1.177	glycerol-3-phosphate 1-dehydrogenase (NADP+)
ec: 1.1.1.178	3-hydroxy-2-methylbutyryl-CoA dehydrogenase
ec: 1.1.1.185	L-glycol dehydrogenase
ec: 1.1.1.190	indole-3-acetaldehyde reductase (NADH)
ec: 1.1.1.191	indole-3-acetaldehyde reductase (NADPH)
ec: 1.1.1.192	long-chain-alcohol dehydrogenase
ec: 1.1.1.194	coniferyl-alcohol dehydrogenase
ec: 1.1.1.195	cinnamyl-alcohol dehydrogenase
ec: 1.1.1.198	(+)-borneol dehydrogenase
ec: 1.1.1.202	1,3-propanediol dehydrogenase
ec: 1.1.1.207	(−)-menthol dehydrogenase
ec: 1.1.1.208	(+)-neomenthol dehydrogenase
ec: 1.1.1.216	farnesol dehydrogenase
ec: 1.1.1.217	benzyl-2-methyl-hydroxybutyrate dehydrogenase
ec: 1.1.1.222	(R)-4-hydroxyphenyllactate dehydrogenase
ec: 1.1.1.223	isopiperitenol dehydrogenase
ec: 1.1.1.226	4-hydroxycyclohexanecarboxylate dehydrogenase
ec: 1.1.1.229	diethyl 2-methyl-3-oxosuccinate reductase
ec: 1.1.1.237	hydroxyphenylpyruvate reductase
ec: 1.1.1.244	methanol dehydrogenase
ec: 1.1.1.245	cyclohexanol dehydrogenase
ec: 1.1.1.250	D-arabinitol 2-dehydrogenase
ec: 1.1.1.251	galactitol 1-phosphate 5-dehydrogenase
ec: 1.1.1.255	mannitol dehydrogenase
ec: 1.1.1.256	fluoren-9-ol dehydrogenase
ec: 1.1.1.257	4-(hydroxymethyl)benzenesulfonate dehydrogenase
ec: 1.1.1.258	6-hydroxyhexanoate dehydrogenase
ec: 1.1.1.259	3-hydroxypimeloyl-CoA dehydrogenase
ec: 1.1.1.261	glycerol-1-phosphate dehydrogenase [NAD(P)+]
ec: 1.1.1.265	3-methylbutanal reductase
ec: 1.1.1.283	methylglyoxal reductase (NADPH-dependent)
ec: 1.1.1.286	isocitrate-homoisocitrate dehydrogenase
ec: 1.1.1.287	D-arabinitol dehydrogenase (NADP+)
	butanol dehydrogenase
	ALDEHYDE DEHYDROGENASES
ec: 1.2.1.2	formate dehydrogenase
ec: 1.2.1.3	aldehyde dehydrogenase (NAD+)

TABLE 1-continued

Alcohol and Aldehyde Dehydrogenases for Conversion of 4-HB to BDO.

ec: 1.2.1.4	aldehyde dehydrogenase (NADP+)
ec: 1.2.1.5	aldehyde dehydrogenase [NAD(P)+]
ec: 1.2.1.7	benzaldehyde dehydrogenase (NADP+)
ec: 1.2.1.8	betaine-aldehyde dehydrogenase
ec: 1.2.1.9	glyceraldehyde-3-phosphate dehydrogenase (NADP+)
ec: 1.2.1.10	acetaldehyde dehydrogenase (acetylating)
ec: 1.2.1.11	aspartate-semialdehyde dehydrogenase
ec: 1.2.1.12	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
ec: 1.2.1.13	glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating)
ec: 1.2.1.15	malonate-semialdehyde dehydrogenase
ec: 1.2.1.16	succinate-semialdehyde dehydrogenase [NAD(P)+]
ec: 1.2.1.17	glyoxylate dehydrogenase (acylating)
ec: 1.2.1.18	malonate-semialdehyde dehydrogenase (acetylating)
ec: 1.2.1.19	aminobutyraldehyde dehydrogenase
ec: 1.2.1.20	glutarate-semialdehyde dehydrogenase
ec: 1.2.1.21	glycolaldehyde dehydrogenase
ec: 1.2.1.22	lactaldehyde dehydrogenase
ec: 1.2.1.23	2-oxoaldehyde dehydrogenase (NAD+)
ec: 1.2.1.24	succinate-semialdehyde dehydrogenase
ec: 1.2.1.25	2-oxoisovalerate dehydrogenase (acylating)
ec: 1.2.1.26	2,5-dioxovalerate dehydrogenase
ec: 1.2.1.27	methylmalonate-semialdehyde dehydrogenase (acylating)
ec: 1.2.1.28	benzaldehyde dehydrogenase (NAD+)
ec: 1.2.1.29	aryl-aldehyde dehydrogenase
ec: 1.2.1.30	aryl-aldehyde dehydrogenase (NADP+)
ec: 1.2.1.31	L-aminoadipate-semialdehyde dehydrogenase
ec: 1.2.1.32	aminomuconate-semialdehyde dehydrogenase
ec: 1.2.1.36	retinal dehydrogenase
ec: 1.2.1.39	phenylacetaldehyde dehydrogenase
ec: 1.2.1.41	glutamate-5-semialdehyde dehydrogenase
ec: 1.2.1.42	hexadecanal dehydrogenase (acylating)
ec: 1.2.1.43	formate dehydrogenase (NADP+)
ec: 1.2.1.45	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase
ec: 1.2.1.46	formaldehyde dehydrogenase
ec: 1.2.1.47	4-trimethylammoniobutyraldehyde dehydrogenase
ec: 1.2.1.48	long-chain-aldehyde dehydrogenase
ec: 1.2.1.49	2-oxoaldehyde dehydrogenase (NADP+)
ec: 1.2.1.51	pyruvate dehydrogenase (NADP+)
ec: 1.2.1.52	oxoglutarate dehydrogenase (NADP+)
ec: 1.2.1.53	4-hydroxyphenylacetaldehyde dehydrogenase
ec: 1.2.1.57	butanal dehydrogenase
ec: 1.2.1.58	phenylglyoxylate dehydrogenase (acylating)
ec: 1.2.1.59	glyceraldehyde-3-phosphate dehydrogenase (NAD(P)+) (phosphorylating)
ec: 1.2.1.62	4-formylbenzenesulfonate dehydrogenase
ec: 1.2.1.63	6-oxohexanoate dehydrogenase
ec: 1.2.1.64	4-hydroxybenzaldehyde dehydrogenase
ec: 1.2.1.65	salicylaldehyde dehydrogenase
ec: 1.2.1.66	mycothiol-dependent formaldehyde dehydrogenase
ec: 1.2.1.67	vanillin dehydrogenase
ec: 1.2.1.68	coniferyl-aldehyde dehydrogenase
ec: 1.2.1.69	fluoroacetaldehyde dehydrogenase
ec: 1.2.1.71	succinylglutamate-semialdehyde dehydrogenase

Other exemplary enzymes and pathways are disclosed herein (see Examples). Furthermore, it is understood that enzymes can be utilized for carry out reactions for which the substrate is not the natural substrate. While the activity for the non-natural substrate may be lower than the natural substrate, it is understood that such enzymes can be utilized, either as naturally occurring or modified using the directed evolution or adaptive evolution, as disclosed herein (see also Examples).

BDO production through any of the pathways disclosed herein are based, in part, on the identification of the appropriate enzymes for conversion of precursors to BDO. A number of specific enzymes for several of the reaction steps have been identified. For those transformations where enzymes specific to the reaction precursors have not been identified, enzyme candidates have been identified that are best suited for catalyzing the reaction steps. Enzymes have been shown to operate on a broad range of substrates, as discussed below. In addition, advances in the field of protein engineering also make it feasible to alter enzymes to act efficiently on substrates, even if not a natural substrate. Described below are several examples of broad-specificity enzymes from diverse classes suitable for a BDO pathway as well as methods that have been used for evolving enzymes to act on non-natural substrates.

A key class of enzymes in BDO pathways is the oxidoreductases that interconvert ketones or aldehydes to alcohols (1.1.1). Numerous exemplary enzymes in this class can operate on a wide range of substrates. An alcohol dehydrogenase (1.1.1.1) purified from the soil bacterium *Brevibacterium* sp KU 1309 (Hirano et al., *J. Biosc. Bioeng.* 100: 318-322 (2005)) was shown to operate on a plethora of aliphatic as well as aromatic alcohols with high activities. Table 2 shows the activity of the enzyme and its K_m on different alcohols. The enzyme is reversible and has very high activity on several aldehydes also (Table 3).

TABLE 2

Relative activities of an alcohol dehydrogenase from *Brevibacterium* sp KU to oxidize various alcohols.

Substrate	Relative Activity (0%)	K_m (mM)
2-Phenylethanol	100*	0.025
(S)-2-Phenylpropanol	156	0.157
(R)-2-Phenylpropanol	63	0.020
Bynzyl alcohol	199	0.012
3-Phenylpropanol	135	0.033
Ethanol	76	
1-Butanol	111	
1-Octanol	101	
1-Dodecanol	68	
1-Phenylethanol	46	
2-Propanol	54	

*The activity of 2-phenylethanol, corresponding to 19.2 U/mg, was taken as 100%.

TABLE 3

Relative activities of an alcohol dehydrogenase from *Brevibacterium* sp KU 1309 to reduce various carbonyl compounds.

Substrate	Relative Activity (%)	K_m (mM)
Phenylacetaldehyde	100	0.261
2-Phenylpropionaldehyde	188	0.864
1-Octylaldehyde	87	
Acetophenone	0	

Lactate dehydrogenase (1.1.1.27) from *Ralstonia eutropha* is another enzyme that has been demonstrated to have high activities on several 2-oxoacids such as 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (a C5 compound analogous to 2-oxoadipate) (Steinbuchel and Schlegel, *Eur. J. Biochem.* 130:329-334 (1983)). Column 2 in Table 4 demonstrates the activities of ldhA from *R. eutropha* (formerly *A. eutrophus*) on different substrates (Steinbuchel and Schlegel, supra, 1983).

TABLE 4

The in vitro activity of *R. eutropha* ldhA (Steinbuchel and Schlegel, supra, 1983) on different substrates and compared with that on pyruvate.

	Activity (%) of		
Substrate	L(+)-lactate dehydrogenase from *A. eutrophus*	L(+)-lactate dehydrogenase from rabbit muscle	D(−)-lactate dehydrogenase from *L. leichmanii*
Glyoxylate	8.7	23.9	5.0
Pyruvate	100.0	100.0	100.0
2-Oxobutyrate	107.0	18.6	1.1
2-Oxovalerate	125.0	0.7	0.0
3-Methyl-2-oxobutyrate	28.5	0.0	0.0
3-Methyl-2-oxovalerate	5.3	0.0	0.0
4-Methyl-2-oxopentanoate	39.0	1.4	1.1
Oxaloacetate	0.0	33.1	23.1
2-Oxoglutarate	79.6	0.0	0.0
3-Fluoropyruvate	33.6	74.3	40.0

Oxidoreductases that can convert 2-oxoacids to their acyl-CoA counterparts (1.2.1) have been shown to accept multiple substrates as well. For example, branched-chain 2-keto-acid dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase (1.2.1.25), participates in branched-chain amino acid degradation pathways, converting 2-keto acids derivatives of valine, leucine and isoleucine to their acyl-CoA derivatives and CO_2. In some organisms including *Rattus norvegicus* (Paxton et al., *Biochem. J.* 234:295-303 (1986)) and *Saccharomyces cerevisiae* (Sinclair et al., *Biochem. Mol. Biol. Int.* 32:911-922 (1993), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanoate and alpha-ketoglutarate, in addition to the branched-chain amino acid precursors.

Members of yet another class of enzymes, namely aminotransferases (2.6.1), have been reported to act on multiple substrates. Aspartate aminotransferase (aspAT) from *Pyrococcus furiosus* has been identified, expressed in *E. coli* and the recombinant protein characterized to demonstrate that the enzyme has the highest activities towards aspartate and alpha-ketoglutarate but lower, yet significant activities towards alanine, glutamate and the aromatic amino acids (Ward et al., *Archaea* 133-141 (2002)). In another instance, an aminotransferase identified from *Leishmania mexicana* and expressed in *E. coli* (Vernal et al., *FEMS Microbiol. Lett.* 229:217-222 (2003)) was reported to have a broad substrate specificity towards tyrosine (activity considered 100% on tyrosine), phenylalanine (90%), tryptophan (85%), aspartate (30%), leucine (25%) and methionine (25%), respectively (Vernal et al., *Mol. Biochem. Parasitol* 96:83-92 (1998)). Similar broad specificity has been reported for a tyrosine aminotransferase from *Trypanosoma cruzi*, even though both of these enzymes have a sequence homology of only 6%. The latter enzyme can accept leucine, methionine as well as tyrosine, phenylalanine, tryptophan and alanine as efficient amino donors (Nowicki et al., *Biochim. Biophys. Acta* 1546: 268-281 (2001)).

CoA transferases (2.8.3) have been demonstrated to have the ability to act on more than one substrate. Specifically, a CoA transferase was purified from *Clostridium acetobutylicum* and was reported to have the highest activities on acetate, propionate, and butyrate. It also had significant activities with valerate, isobutyrate, and crotonate (Wiesenborn et al., *Appl. Environ. Microbiol.* 55:323-329 (1989)). In another study, the *E. coli* enzyme acyl-CoA:acetate-CoA transferase, also known as acetate-CoA transferase (EC 2.8.3.8), has been shown to transfer the CoA moiety to acetate from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Matthies and Schink, *App. Environm. Microbiol.* 58:1435-1439 (1992)), valerate (Vanderwinkel et al., *Biochem. Biophys. Res Commun.* 33:902-908 (1968b)) and butanoate (Vanderwinkel et al., *Biochem. Biophys. Res Commun.* 33:902-908(1968a).

Other enzyme classes additionally support broad substrate specificity for enzymes. Some isomerases (5.3.3) have also been proven to operate on multiple substrates. For example, L-rhamnose isomerase from *Pseudomonas stutzeri* catalyzes the isomerization between various aldoalses and ketoses (Yoshida et al., *J. Mol. Biol.* 365:1505-1516 (2007)). These include isomerization between L-rhamnose and L-rhamnulose, L-mannose and L-fructose, L-xylose and L-xylulose, D-ribose and D-ribulose, and D-allose and D-psicose.

In yet another class of enzymes, the phosphotransferases (2.7.1), the homoserine kinase (2.7.1.39) from *E. coli* that converts L-homoserine to L-homoserine phosphate, was found to phosphorylate numerous homoserine analogs. In these substrates, the carboxyl functional group at the R-position had been replaced by an ester or by a hydroxymethyl group (Huo and Viola, *Biochemistry* 35:16180-16185 (1996)). Table 5 demonstrates the broad substrate specificity of this kinase.

TABLE 5

The substrate specificity of homoserine kinase.

Substrate	k_{cat}	% k_{cat}	K_m (mM)	k_{cat}/K_m
L-homoserine	18.3 ± 0.1	100	0.14 ± 0.04	184 ± 17
D-homoserine	8.3 ± 1.1	32	31.8 ± 7.2	0.26 ± 0.03
L-aspartate β-semialdehyde	2.1 ± 0.1	8.2	0.28 ± 0.02	7.5 ± 0.3
L-2-amino-1,4-butanediol	2.0 ± 0.5	7.9	11.6 ± 6.5	0.17 ± 0.06
L-2-amino-5-hydroxyvalerate	2.5 ± 0.4	9.9	1.1 ± 0.5	2.3 ± 0.3
L-homoserine methyl ester	14.7 ± 2.6	80	4.9 ± 2.0	3.0 ± 0.6
L-homoserine ethyl ester	13.6 ± 0.8	74	1.9 ± 0.5	7.2 ± 1.7
L-homoserine isopropyl ester	13.6 ± 1.4	74	1.2 ± 0.5	11.3 ± 1.1

TABLE 5-continued

The substrate specificity of homoserine kinase.

Substrate	k_{cat}	% k_{cat}	K_m (mM)	k_{cat}/K_m
L-homoserine n-propyl ester	14.0 ± 0.4	76	3.5 ± 0.4	4.0 ± 1.2
L-homoserine isobutyl ester	16.4 ± 0.8	84	6.9 ± 1.1	2.4 ± 0.3
L-homserine n-butyl ester	29.1 ± 1.2	160	5.8 ± 0.8	5.0 ± 0.5

Another class of enzymes useful in BDO pathways is the acid-thiol ligases (6.2.1). Like enzymes in other classes, certain enzymes in this class have been determined to have broad substrate specificity. For example, acyl CoA ligase from *Pseudomonas putida* has been demonstrated to work on several aliphatic substrates including acetic, propionic, butyric, valeric, hexanoic, heptanoic, and octanoic acids and on aromatic compounds such as phenylacetic and phenoxy-acetic acids (Fernandez-Valverde et al., *Appl. Environ. Microbiol.* 59:1149-1154 (1993)). A related enzyme, malonyl CoA synthetase (6.3.4.9) from *Rhizobium trifolii* could convert several diacids, namely, ethyl-, propyl-, allyl-, isopropyl-, dimethyl-, cyclopropyl-, cyclopropylmethylene-, cyclobutyl-, and benzyl-malonate into their corresponding monothioesters (Pohl et al., *J. Am. Chem. Soc.* 123:5822-5823 (2001)). Similarly, decarboxylases (4.1.1) have also been found with broad substrate ranges. Pyruvate decarboxylase (PDC), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. The enzyme isolated from *Saccharomyces cerevisiae* has a broad substrate range for aliphatic 2-keto acids including 2-ketobutyrate, 2-ketovalerate, and 2-phenylpyruvate (Li and Jordan, *Biochemistry* 38:10004-10012 (1999)). Similarly, benzoylformate decarboxylase has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Polovnikova et al., *Biochemistry* 42:1820-1830 (2003); Hasson et al., *Biochemistry* 37:9918-9930 (1998)). Branched chain alpha-ketoacid decarboxylase (BCKA) has been shown to act on a variety of compounds varying in chain length from 3 to 6 carbons (Oku and Kaneda, *J. Biol. Chem.* 263:18386-18396 (1998); Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005b)). The enzyme in *Lactococcus lactis* has been characterized on a variety of branched and linear substrates including 2-oxobutanoate, 2-oxohexanoate, 2-oxopentanoate, 3-methyl-2-oxobutanoate, 4-methyl-2-oxobutanoate and isocaproate (Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005a).

Interestingly, enzymes known to have one dominant activity have also been reported to catalyze a very different function. For example, the cofactor-dependent phosphoglycerate mutase (5.4.2.1) from *Bacillus stearothermophilus* and *Bacillus subtilis* is known to function as a phosphatase as well (Rigden et al., *Protein Sci.* 10:1835-1846 (2001)). The enzyme from *B. stearothermophilus* is known to have activity on several substrates, including 3-phosphoglycerate, alpha-napthylphosphate, p-nitrophenylphosphate, AMP, fructose-6-phosphate, ribose-5-phosphate and CMP.

In contrast to these examples where the enzymes naturally have broad substrate specificities, numerous enzymes have been modified using directed evolution to broaden their specificity towards their non-natural substrates. Alternatively, the substrate preference of an enzyme has also been changed using directed evolution. Therefore, it is feasible to engineer a given enzyme for efficient function on a natural, for example, improved efficiency, or a non-natural substrate, for example, increased efficiency. For example, it has been reported that the enantioselectivity of a lipase from *Pseudomonas aeruginosa* was improved significantly (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 36:2830-2832 (1997)). This enzyme hydrolyzed p-nitrophenyl 2-methyldecanoate with only 2% enantiomeric excess (ee) in favor of the (S)-acid. However, after four successive rounds of error-prone mutagenesis and screening, a variant was produced that catalyzed the requisite reaction with 81% ee (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 36:2830-2832 (1997)).

Directed evolution methods have been used to modify an enzyme to function on an array of non-natural substrates. The substrate specificity of the lipase in *P. aeruginosa* was broadened by randomization of amino acid residues near the active site. This allowed for the acceptance of alpha-substituted carboxylic acid esters by this enzyme (Reetz et al., *Agnew. Chem. Int. Ed Engl.* 44:4192-4196 (2005)). In another successful modification of an enzyme, DNA shuffling was employed to create an *Escherichia coli* aminotransferase that accepted β-branched substrates, which were poorly accepted by the wild-type enzyme (Yano et al., *Proc. Nat. Acad. Sci. U.S.A.* 95:5511-5515 (1998)). Specifically, at the end of four rounds of shuffling, the activity of aspartate aminotransferase for valine and 2-oxovaline increased by up to five orders of magnitude, while decreasing the activity towards the natural substrate, aspartate, by up to 30-fold. Recently, an algorithm was used to design a retro-aldolase that could be used to catalyze the carbon-carbon bond cleavage in a non-natural and non-biological substrate, 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone (Jiang et al., *Science* 319:1387-1391 (2008)). These algorithms used different combinations of four different catalytic motifs to design new enzyme, and 20 of the selected designs for experimental characterization had four-fold improved rates over the uncatalyzed reaction (Jiang et al., *Science* 319:1387-1391 (2008)). Thus, not only are these engineering approaches capable of expanding the array of substrates on which an enzyme can act, but they allow the design and construction of very efficient enzymes. For example, a method of DNA shuffling (random chimeragenesis on transient templates or RACHITT) was reported to lead to an engineered monooxygenase that had an improved rate of desulfurization on complex substrates as well as 20-fold faster conversion of a non-natural substrate (Coco et al., *Nat. Biotechnol.* 19:354-359 (2001)). Similarly, the specific activity of a sluggish mutant triosephosphate isomerase enzyme was improved up to 19-fold from 1.3 fold (Hermes et al., *Proc. Nat. Acad. Sci. U.S.A.* 87:696-700 1990)). This enhancement in specific activity was accomplished by using random mutagenesis over the whole length of the protein and the improvement could be traced back to mutations in six amino acid residues.

The effectiveness of protein engineering approaches to alter the substrate specificity of an enzyme for a desired substrate has also been demonstrated in several studies. Isopropylmalate dehydrogenase from *Thermus thermophilus* was modified by changing residues close to the active site so that it could now act on malate and D-lactate as substrates (Fujita et al., *Biosci. Biotechnol. Biochem.* 65:2695-2700 (2001)). In this study as well as in others, it was pointed out that one or a few residues could be modified to alter the substrate specificity. For example, the dihydroflavonol 4-reductase for which a single amino acid was changed in the presumed substrate-binding region could

preferentially reduce dihydrokaempferol (Johnson et al., *Plant. J.* 25:325-333 (2001)). The substrate specificity of a very specific isocitrate dehydrogenase from *Escherichia coli* was changed form isocitrate to isopropylmalate by changing one residue in the active site (Doyle et al., *Biochemistry* 40:4234-4241 (2001)). Similarly, the cofactor specificity of a NAD^+-dependent 1,5-hydroxyprostaglandin dehydrogenase was altered to $NADP^+$ by changing a few residues near the N-terminal end (Cho et al., *Arch. Biochem. Biophys.* 419:139-146 (2003)). Sequence analysis and molecular modeling analysis were used to identify the key residues for modification, which were further studied by site-directed mutagenesis.

Numerous examples exist spanning diverse classes of enzymes where the function of enzyme was changed to favor one non-natural substrate over the natural substrate of the enzyme. A fucosidase was evolved from a galactosidase in *E. coli* by DNA shuffling and screening (Zhang et al., *Proc. Natl. Acad. Sci. U.S.A.* 94:4504-4509 (1997)). Similarly, aspartate aminotransferase from *E. coli* was converted into a tyrosine aminotransferase using homology modeling and site-directed mutagenesis (Onuffer and Kirsch, *Protein Sci.*, 4:1750-1757 (1995)). Site-directed mutagenesis of two residues in the active site of benzoylformate decarboxylase from *P. putida* reportedly altered the affinity (K_m) towards natural and non-natural substrates (Siegert et al., *Protein Eng Des Sel* 18:345-357 (2005)). Cytochrome c peroxidase (CCP) from *Saccharomyces cerevisiae* was subjected to directed molecular evolution to generate mutants with increased activity against the classical peroxidase substrate guaiacol, thus changing the substrate specificity of CCP from the protein cytochrome c to a small organic molecule. After three rounds of DNA shuffling and screening, mutants were isolated which possessed a 300-fold increased activity against guaiacol and up to 1000-fold increased specificity for this substrate relative to that for the natural substrate (Iffland et al., *Biochemistry* 39:10790-10798 (2000)).

In some cases, enzymes with different substrate preferences than either of the parent enzymes have been obtained. For example, biphenyl-dioxygenase-mediated degradation of polychlorinated biphenyls was improved by shuffling genes from two bacteria, *Pseudomonas pseudoalcaligens* and *Burkholderia cepacia* (Kumamaru et al., *Nat. Biotechnol.* 16:663-666 (1998)). The resulting chimeric biphenyl oxygenases showed different substrate preferences than both the parental enzymes and enhanced the degradation activity towards related biphenyl compounds and single aromatic ring hydrocarbons such as toluene and benzene which were originally poor substrates for the enzyme.

In addition to changing enzyme specificity, it is also possible to enhance the activities on substrates for which the enzymes naturally have low activities. One study demonstrated that amino acid racemase from *P. putida* that had broad substrate specificity (on lysine, arginine, alanine, serine, methionine, cysteine, leucine and histidine among others) but low activity towards tryptophan could be improved significantly by random mutagenesis (Kino et al., *Appl. Microbiol. Biotechnol.* 73:1299-1305 (2007)). Similarly, the active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, *Biochemistry* 33:12879-12885 (1994)). An interesting aspect of these approaches is that even if random methods have been applied to generate these mutated enzymes with efficacious activities, the exact mutations or structural changes that confer the improvement in activity can be identified. For example, in the aforementioned study, the mutations that facilitated improved activity on tryptophan was traced back to two different positions.

Directed evolution has also been used to express proteins that are difficult to express. For example, by subjecting horseradish peroxidase to random mutagenesis and gene recombination, mutants were identified that had more than 14-fold higher activity than the wild type (Lin et al., *Biotechnol. Prog.* 15:467-471 (1999)).

Another example of directed evolution shows the extensive modifications to which an enzyme can be subjected to achieve a range of desired functions. The enzyme lactate dehydrogenase from *Bacillus stearothermophilus* was subjected to site-directed mutagenesis, and three amino acid substitutions were made at sites that were believed to determine the specificity towards different hydroxyacids (Clarke et al., *Biochem. Biophys. Res. Commun.* 148:15-23 (1987)). After these mutations, the specificity for oxaloacetate over pyruvate was increased to 500 in contrast to the wild type enzyme that had a catalytic specificity for pyruvate over oxaloacetate of 1000. This enzyme was further engineered using site-directed mutagenesis to have activity towards branched-chain substituted pyruvates (Wilks et al., *Biochemistry* 29:8587-8591 (1990)). Specifically, the enzyme had a 55-fold improvement in K_{cat} for alpha-ketoisocaproate. Three structural modifications were made in the same enzyme to change its substrate specificity from lactate to malate. The enzyme was highly active and specific towards malate (Wilks et al., *Science* 242:1541-1544 (1988)). The same enzyme from *B. stearothermophilus* was subsequently engineered to have high catalytic activity towards alpha-keto acids with positively charged side chains, such as those containing ammonium groups (Hogan et al., *Biochemistry* 34:4225-4230 (1995)). Mutants with acidic amino acids introduced at position 102 of the enzyme favored binding of such side chain ammonium groups. The results obtained proved that the mutants showed up to 25-fold improvements in k_{cat}/K_m values for omega-amino-alpha-keto acid substrates. Interestingly, this enzyme was also structurally modified to function as a phenyllactate dehydrogenase instead of a lactate dehydrogenase (Wilks et al., *Biochemistry* 31:7802-7806 1992). Restriction sites were introduced into the gene for the enzyme which allowed a region of the gene to be excised. This region coded for a mobile surface loop of the polypeptide (residues 98-110) which normally seals the active site from bulk solvent and is a major determinant of substrate specificity. The variable length and sequence loops were inserted so that hydroxyacid dehydrogenases with altered substrate specificities were generated. With one longer loop construction, activity with pyruvate was reduced one-million-fold but activity with phenylpyruvate was largely unaltered. A switch in specificity (k_{at}/K_m) of 390.000-fold was achieved. The 1700:1 selectivity of this enzyme for phenylpyruvate over pyruvate is that required in a phenyllactate dehydrogenase. The studies described above indicate that various approaches of enzyme engineering can be used to obtain enzymes for the BDO pathways as disclosed herein.

As disclosed herein, biosynthetic pathways to 1,4-butanediol from a number of central metabolic intermediates are can be utilized, including acetyl-CoA, succinyl-CoA, alpha-ketoglutarate, glutamate, 4-aminobutyrate, and homoserine. Acetyl-CoA, succinyl-CoA and alpha-ketoglutarate are common intermediates of the tricarboxylic acid (TCA) cycle, a series of reactions that is present in its entirety in nearly all living cells that utilize oxygen for cellular respiration and is present in truncated forms in a number of anaerobic organisms. Glutamate is an amino acid that is

derived from alpha-ketoglutarate via glutamate dehydrogenase or any of a number of transamination reactions (see FIG. **8**B). 4-aminobutyrate can be formed by the decarboxylation of glutamate (see FIG. **8**B) or from acetoacetyl-CoA via the pathway disclosed in FIG. **9**C. Acetoacetyl-CoA is derived from the condensation of two acetyl-CoA molecules by way of the enzyme, acetyl-coenzyme A acetyltransferase, or equivalently, acetoacetyl-coenzyme A thiolase. Homoserine is an intermediate in threonine and methionine metabolism, formed from oxaloacetate via aspartate. The conversion of oxaloacetate to homoserine requires one NADH, two NADPH, and one ATP.

Pathways other than those exemplified above also can be employed to generate the biosynthesis of BDO in non-naturally occurring microbial organisms. In one embodiment, biosynthesis can be achieved using a L-homoserine to BDO pathway (see FIG. **13**). This pathway has a molar yield of 0.90 mol/mol glucose, which appears restricted by the availability of reducing equivalents. A second pathway synthesizes BDO from acetoacetyl-CoA and is capable of achieving the maximum theoretical yield of 1.091 mol/mol glucose (see FIG. **9**). Implementation of either pathway can be achieved by introduction of two exogenous enzymes into a host organism such as *E. coli*, and both pathways can additionally complement BDO production via succinyl-CoA. Pathway enzymes, thermodynamics, theoretical yields and overall feasibility are described further below.

A homoserine pathway also can be engineered to generate BDO-producing microbial organisms. Homoserine is an intermediate in threonine and methionine metabolism, formed from oxaloacetate via aspartate. The conversion of oxaloacetate to homoserine requires one NADH, two NADPH, and one ATP (FIG. **2**). Once formed, homoserine feeds into biosynthetic pathways for both threonine and methionine. In most organisms, high levels of threonine or methionine feedback to repress the homoserine biosynthesis pathway (Caspi et al., *Nucleic Acids Res.* 34:D511-D516 (1990)).

The transformation of homoserine to 4-hydroxybutyrate (4-HB) can be accomplished in two enzymatic steps as described herein. The first step of this pathway is deamination of homoserine by a putative ammonia lyase. In step 2, the product alkene, 4-hydroxybut-2-enoate is reduced to 4-HB by a putative reductase at the cost of one NADH. 4-HB can then be converted to BDO.

Enzymes available for catalyzing the above transformations are disclosed herein. For example, the ammonia lyase in step 1 of the pathway closely resembles the chemistry of aspartate ammonia-lyase (aspartase). Aspartase is a widespread enzyme in microorganisms, and has been characterized extensively (Viola, R. E., *Mol. Biol.* 74:295-341 (2008)). The crystal structure of the *E. coli* aspartase has been solved (Shi et al., *Biochemistry* 36:9136-9144 (1997)), so it is therefore possible to directly engineer mutations in the enzyme's active site that would alter its substrate specificity to include homoserine. The oxidoreductase in step 2 has chemistry similar to several well-characterized enzymes including fumarate reductase in the *E. coli* TCA cycle. Since the thermodynamics of this reaction are highly favorable, an endogenous reductase with broad substrate specificity will likely be able to reduce 4-hydroxybut-2-enoate. The yield of this pathway under anaerobic conditions is 0.9 mol BDO per mol glucose.

The succinyl-CoA pathway was found to have a higher yield due to the fact that it is more energetically efficient. The conversion of one oxaloacetate molecule to BDO via the homoserine pathway will require the expenditure of 2 ATP equivalents. Because the conversion of glucose to two oxaloacetate molecules can generate a maximum of 3 ATP molecules assuming PEP carboxykinase to be reversible, the overall conversion of glucose to BDO via homoserine has a negative energetic yield. As expected, if it is assumed that energy can be generated via respiration, the maximum yield of the homoserine pathway increases to 1.05 mol/mol glucose which is 96% of the succinyl-CoA pathway yield. The succinyl-CoA pathway can channel some of the carbon flux through pyruvate dehydrogenase and the oxidative branch of the TCA cycle to generate both reducing equivalents and succinyl-CoA without an energetic expenditure. Thus, it does not encounter the same energetic difficulties as the homoserine pathway because not all of the flux is channeled through oxaloacetate to succinyl-CoA to BDO. Overall, the homoserine pathway demonstrates a high-yielding route to BDO.

An acetoacetate pathway also can be engineered to generate BDO-producing microbial organisms. Acetoacetate can be formed from acetyl-CoA by enzymes involved in fatty acid metabolism, including acetyl-CoA acetyltransferase and acetoacetyl-CoA transferase. Biosynthetic routes through acetoacetate are also particularly useful in microbial organisms that can metabolize single carbon compounds such as carbon monoxide, carbon dioxide or methanol to form acetyl-CoA.

A three step route from acetoacetyl-CoA to 4-aminobutyrate (see FIG. **9**C) can be used to synthesize BDO through acetoacetyl-CoA. 4-Aminobutyrate can be converted to succinic semialdehyde as shown in FIG. **8**B. Succinic semialdehyde, which is one reduction step removed from succinyl-CoA or one decarboxylation step removed from α-ketoglutarate, can be converted to BDO following three reductions steps (FIG. **1**). Briefly, step 1 of this pathway involves the conversion of acetoacetyl-CoA to acetoacetate by, for example, the *E. coli* acetoacetyl-CoA transferase encoded by the atoA and atoD genes (Hanai et al., *Appl. Environ. Microbiol.* 73: 7814-7818 (2007)). Step 2 of the acetoacetyl-CoA biopathway entails conversion of acetoacetate to 3-aminobutanoate by an ω-aminotransferase. The ω-amino acid:pyruvate aminotransferase (ω-APT) from *Alcaligens denitrificans* was overexpressed in *E. coli* and shown to have a high activity toward 3-aminobutanoate in vitro (Yun et al., *Appl. Environ. Microbiol.* 70:2529-2534 (2004)).

In step 2, a putative aminomutase shifts the amine group from the 3- to the 4-position of the carbon backbone. An aminomutase performing this function on 3-aminobutanoate has not been characterized, but an enzyme from *Clostridium sticklandii* has a very similar mechanism. The enzyme, D-lysine-5,6-aminomutase, is involved in lysine biosynthesis.

The synthetic route to BDO from acetoacetyl-CoA passes through 4-aminobutanoate, a metabolite in *E. coli* that's normally formed from decarboxylation of glutamate. Once formed, 4-aminobutanoate can be converted to succinic semialdehyde by 4-aminobutanoate transaminase (2.6.1.19), an enzyme which has been biochemically characterized.

One consideration for selecting candidate enzymes in this pathway is the stereoselectivity of the enzymes involved in steps 2 and 3. The ω-ABT in *Alcaligens denitrificans* is specific to the L-stereoisomer of 3-aminobutanoate, while D-lysine-5,6-aminomutase likely requires the D-stereoisomer. If enzymes with complementary stereoselectivity are not initially found or engineered, a third enzyme can be added to the pathway with racemase activity that can convert L-3-aminobutanoate to D-3-aminobutanoate. While amino

acid racemases are widespread, whether these enzymes can function on ω-amino acids is not known.

The maximum theoretical molar yield of this pathway under anaerobic conditions is 1.091 mol/mol glucose. In order to generate flux from acetoacetyl-CoA to BDO it was necessary to assume that acetyl-CoA:acetoacetyl-CoA transferase is reversible. The function of this enzyme in *E. coli* is to metabolize short-chain fatty acids by first converting them into thioesters.

While the operation of acetyl-CoA:acetoacetyl-CoA transferase in the acetate-consuming direction has not been demonstrated experimentally in *E. coli*, studies on similar enzymes in other organisms support the assumption that this reaction is reversible. The enzyme butyryl-CoA:acetate: CoA transferase in gut microbes *Roseburia* sp. and *F. prasnitzii* operates in the acetate utilizing direction to produce butyrate (Duncan et al., *Appl. Environ. Microbiol.* 68:5186-5190 (2002)). Another very similar enzyme, acetyl: succinate CoA-transferase in *Trypanosoma brucei*, also operates in the acetate utilizing direction. This reaction has a $\Delta_{rxn}G$ close to equilibrium, so high concentrations of acetate can likely drive the reaction in the direction of interest. At the maximum theoretical BDO production rate of 1.09 mol/mol glucose simulations predict that *E. coli* can generate 1.098 mol ATP per mol glucose with no fermentation byproducts. This ATP yield should be sufficient for cell growth, maintenance, and production. The acetoacetatyl-CoA biopathway is a high-yielding route to BDO from acetyl-CoA.

Therefore, in addition to any of the various modifications exemplified previously for establishing 4-HB biosynthesis in a selected host, the BDO producing microbial organisms can include any of the previous combinations and permutations of 4-HB pathway metabolic modifications as well as any combination of expression for CoA-independent aldehyde dehydrogenase, CoA-dependent aldehyde dehydrogenase or an alcohol dehydrogenase or other enzymes disclosed herein to generate biosynthetic pathways for GBL and/or BDO. Therefore, the BDO producers of the invention can have exogenous expression of, for example, one, two, three, four, five, six, seven, eight, nine, or up to all enzymes corresponding to any of the 4-HB pathway and/or any of the BDO pathway enzymes disclosed herein.

Design and construction of the genetically modified microbial organisms is carried out using methods well known in the art to achieve sufficient amounts of expression to produce BDO. In particular, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of BDO resulting in intracellular concentrations between about 0.1-200 mM or more, such as about 0.1-25 mM or more, as discussed above. For example, the intracellular concentration of BDO is between about 3-20 mM, particularly between about 5-15 mM and more particularly between about 8-12 mM, including about 10 mM or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention. As with the 4-HB producers, the BDO producers also can be sustained, cultured or fermented under anaerobic conditions.

The invention further provides a method for the production of 4-HB. The method includes culturing a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway comprising at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate: succinic semialdehyde transaminase, α-ketoglutarate decarboxylase, or glutamate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB). The method can additionally include chemical conversion of 4-HB to GBL and to BDO or THF, for example.

Additionally provided is a method for the production of 4-HB. The method includes culturing a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway including at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase or α-ketoglutarate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB). The 4-HB product can be secreted into the culture medium.

Further provided is a method for the production of BDO. The method includes culturing a non-naturally occurring microbial biocatalyst or microbial organism, comprising a microbial organism having 4-hydroxybutanoic acid (4-HB) and 1,4-butanediol (BDO) biosynthetic pathways, the pathways including at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, 4-hydroxybutyrate kinase, phosphotranshydroxybutyrylase, α-ketoglutarate decarboxylase, aldehyde dehydrogenase, alcohol dehydrogenase or an aldehyde/alcohol dehydrogenase for a sufficient period of time to produce 1,4-butanediol (BDO). The BDO product can be secreted into the culture medium.

Additionally provided are methods for producing BDO by culturing a non-naturally occurring microbial organism having a BDO pathway of the invention. The BDO pathway can comprise at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA oxidoreductase (deaminating), 4-aminobutyryl-CoA transaminase, or 4-hydroxybutyryl-CoA dehydrogenase (see Example VII and Table 17).

Alternatively, the BDO pathway can compare at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate CoA transferase, 4-aminobutyryl-CoA hydrolase, 4-aminobutyrate-CoA ligase, 4-aminobutyryl-CoA reductase (alcohol forming), 4-aminobutyryl-CoA reductase, 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-ol oxidoreductase (deaminating) or 4-aminobutan-1-ol transaminase (see Example VII and Table 18).

In addition, the invention provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 4-aminobutyrate kinase, 4-aminobutyraldehyde dehydrogenase (phosphorylating), 4-aminobutan-1-ol dehydrogenase, 4-aminobutan-1-oloxidoreductase (deaminating), 4-aminobutan-1-ol transaminase, [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating), [(4-aminobu-

tanolyl)oxy]phosphonic acid transaminase, 4-hydroxybutyryl-phosphate dehydrogenase, or 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (see Example VII and Table 19).

The invention further provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising alpha-ketoglutarate 5-kinase, 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating), 2,5-dioxopentanoic acid reductase, alpha-ketoglutarate CoA transferase, alpha-ketoglutaryl-CoA hydrolase, alpha-ketoglutaryl-CoA ligase, alpha-ketoglutaryl-CoA reductase, 5-hydroxy-2-oxopentanoic acid dehydrogenase, alpha-ketoglutaryl-CoA reductase (alcohol forming), 5-hydroxy-2-oxopentanoic acid decarboxylase, or 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Example VIII and Table 20).

The invention additionally provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising glutamate CoA transferase, glutamyl-CoA hydrolase, glutamyl-CoA ligase, glutamate 5-kinase, glutamate-5-semialdehyde dehydrogenase (phosphorylating), glutamyl-CoA reductase, glutamate-5-semialdehyde reductase, glutamyl-CoA reductase (alcohol forming), 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating), 2-amino-5-hydroxypentanoic acid transaminase, 5-hydroxy-2-oxopentanoic acid decarboxylase, 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)(see Example IX and Table 21).

The invention additionally includes a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, vinylacetyl-CoA A-isomerase, or 4-hydroxybutyryl-CoA dehydratase (see Example X and Table 22).

Also provided is a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising homoserine deaminase, homoserine CoA transferase, homoserine-CoA hydrolase, homoserine-CoA ligase, homoserine-CoA deaminase, 4-hydroxybut-2-enoyl-CoA transferase, 4-hydroxybut-2-enoyl-CoA hydrolase, 4-hydroxybut-2-enoyl-CoA ligase, 4-hydroxybut-2-enoate reductase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, or 4-hydroxybut-2-enoyl-CoA reductase (see Example XI and Table 23).

The invention additionally provides a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating). Such a BDO pathway can further comprise succinyl-CoA reductase, 4-hydroxybutyrate dehydrogenase, 4-hydroxybutyryl-CoA transferase, 4-hydroxybutyrate kinase, phosphotrans-4-hydroxybutyrylase, 4-hydroxybutyryl-CoA reductase, 4-hydroxybutyryl-CoA reductase (alcohol forming), or 1,4-butanediol dehydrogenase.

Also provided is a method for producing BDO, comprising culturing a non-naturally occurring microbial organism having a BDO pathway, the pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO, under conditions and for a sufficient period of time to produce BDO, the BDO pathway comprising glutamate dehydrogenase, 4-aminobutyrate oxidoreductase (deaminating), 4-aminobutyrate transaminase, glutamate decarboxylase, 4-hydroxybutyryl-CoA hydrolase, 4-hydroxybutyryl-CoA ligase, 4-hydroxybutanal dehydrogenase (phosphorylating).

The invention additionally provides methods of producing a desired product using the genetically modified organisms disclosed herein that allow improved production of a desired product such as BDO by increasing the product or decreasing undesirable byproducts. Thus, the invention provides a method for producing 1,4-butanediol (BDO), comprising culturing the non-naturally occurring microbial organisms disclosed herein under conditions and for a sufficient period of time to produce BDO. In one embodiment, the invention provides a method of producing BDO using a non-naturally occurring microbial organism, comprising a microbial organism having a 1,4-butanediol (BDO) pathway comprising at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In one embodiment, the microbial organism is genetically modified to express exogenous succinyl-CoA synthetase (see Example XII). For example, the succinyl-CoA synthetase can be encoded by an *Escherichia coli* sucCD genes.

In another embodiment, the microbial organism is genetically modified to express exogenous alpha-ketoglutarate decarboxylase (see Example XIII). For example, the alpha-ketoglutarate decarboxylase can be encoded by the *Mycobacterium bovis* sucA gene. In still another embodiment, the microbial organism is genetically modified to express exogenous succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase and optionally 4-hydroxybutyryl-CoA/acetyl-CoA transferase (see Example XIII). For example, the succinate semialdehyde dehydrogenase (CoA-dependent), 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA/acetyl-CoA transferase can be encoded by *Porphyromonas gingivalis* W83 genes. In an additional embodiment, the microbial organism is genetically modified to express exogenous butyrate kinase and phosphotransbutyrylase (see Example XIII). For example, the butyrate kinase and phosphotransbutyrylase can be encoded by *Clostridium acetobutilicum* buk1 and ptb genes.

In yet another embodiment, the microbial organism is genetically modified to express exogenous 4-hydroxybutyryl-CoA reductase (see Example XIII). For example, the 4-hydroxybutyryl-CoA reductase can be encoded by *Clostridium beijerinckii* ald gene. Additionally, in an embodiment of the invention, the microbial organism is genetically modified to express exogenous 4-hydroxybutanal reductase (see Example XIII). For example, the 4-hydroxybutanal reductase can be encoded by *Geobacillus*

thermoglucosidasius adh1 gene. In another embodiment, the microbial organism is genetically modified to express exogenous pyruvate dehydrogenase subunits (see Example XIV). For example, the exogenous pyruvate dehydrogenase can be NADH insensitive. The pyruvate dehydrogenase subunit can be encoded by the *Klebsiella pneumonia* lpdA gene. In a particular embodiment, the pyruvate dehydrogenase subunit genes of the microbial organism can be under the control of a pyruvate formate lyase promoter.

In still another embodiment, the microbial organism is genetically modified to disrupt a gene encoding an aerobic respiratory control regulatory system (see Example XV). For example, the disruption can be of the arcA gene. Such an organism can further comprise disruption of a gene encoding malate dehydrogenase. In a further embodiment, the microbial organism is genetically modified to express an exogenous NADH insensitive citrate synthase (see Example XV). For example, the NADH insensitive citrate synthase can be encoded by gltA, such as an R163L mutant of gltA. In still another embodiment, the microbial organism is genetically modified to express exogenous phosphoenolpyruvate carboxykinase (see Example XVI). For example, the phosphoenolpyruvate carboxykinase can be encoded by an *Haemophilus influenza* phosphoenolpyruvate carboxykinase gene. It is understood that strains exemplified herein for improved production of BDO can similarly be used, with appropriate modifications, to produce other desired products, for example, 4-hydroxybutyrate or other desired products disclosed herein.

It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, a 4-HB, BDO, THF or GBL biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer 4-HB, BDO, THF or GBL biosynthetic capability. For example, a non-naturally occurring microbial organism having a 4-HB biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes, such as the combination of 4-hydroxybutanoate dehydrogenase and α-ketoglutarate decarboxylase; 4-hydroxybutanoate dehydrogenase and CoA-independent succinic semialdehyde dehydrogenase; 4-hydroxybutanoate dehydrogenase and CoA-dependent succinic semialdehyde dehydrogenase; CoA-dependent succinic semialdehyde dehydrogenase and succinyl-CoA synthetase; succinyl-CoA synthetase and glutamate decarboxylase, and the like. Thus, it is understood that any combination of two or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example, 4-hydroxybutanoate dehydrogenase, α-ketoglutarate decarboxylase and CoA-dependent succinic semialdehyde dehydrogenase; CoA-independent succinic semialdehyde dehydrogenase and succinyl-CoA synthetase; 4-hydroxybutanoate dehydrogenase, CoA-dependent succinic semialdehyde dehydrogenase and glutamate:succinic semialdehyde transaminase, and so forth, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product.

Similarly, for example, with respect to any one or more exogenous nucleic acids introduced to confer BDO production, a non-naturally occurring microbial organism having a BDO biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes, such as the combination of 4-hydroxybutanoate dehydrogenase and α-ketoglutarate decarboxylase; 4-hydroxybutanoate dehydrogenase and 4-hydroxybutyryl CoA:acetyl-CoA transferase; 4-hydroxybutanoate dehydrogenase and butyrate kinase; 4-hydroxybutanoate dehydrogenase and phosphotransbutyrylase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and aldehyde dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and alcohol dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase and an aldehyde/alcohol dehydrogenase, 4-aminobutyrate-CoA transferase and 4-aminobutyryl-CoA transaminase; 4-aminobutyrate kinase and 4-aminobutan-1-ol oxidoreductase (deaminating), and the like. Thus, it is understood that any combination of two or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example, 4-hydroxybutanoate dehydrogenase, α-ketoglutarate decarboxylase and 4-hydroxybutyryl CoA:acetyl-CoA transferase; 4-hydroxybutanoate dehydrogenase, butyrate kinase and phosphotransbutyrylase; 4-hydroxybutanoate dehydrogenase, 4-hydroxybutyryl CoA:acetyl-CoA transferase and aldehyde dehydrogenase; 4-hydroxybutyryl CoA:acetyl-CoA transferase, aldehyde dehydrogenase and alcohol dehydrogenase; butyrate kinase, phosphotransbutyrylase and an aldehyde/alcohol dehydrogenase; 4-aminobutyryl-CoA hydrolase, 4-aminobutyryl-CoA reductase and 4-amino butan-1-ol transaminase; 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase and 4-hydroxybutyryl-CoA dehydratase, and the like. Similarly, any combination of four, five or more enzymes of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the 4-HB producers can be cultured for the biosynthetic production of 4-HB. The 4-HB can be isolated or be treated as described below to generate GBL, THF and/or BDO. Similarly, the BDO producers can be cultured for the biosynthetic production of BDO. The BDO can be isolated or subjected to further treatments for the chemical synthesis of BDO family compounds, as disclosed herein.

The growth medium can include, for example, any carbohydrate source which can supply a source of carbon to the non-naturally occurring microorganism. Such sources include, for example, sugars such as glucose, sucrose, xylose, arabinose, galactose, mannose, fructose and starch. Other sources of carbohydrate include, for example, renewable feedstocks and biomass. Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulosic biomass, hemicellulosic biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, sucrose, xylose, arabinose, galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those

skilled in the art will understand that renewable feedstocks and biomass other than those exemplified above also can be used for culturing the microbial organisms of the invention for the production of 4-HB or BDO and other compounds of the invention.

Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, 4-HB, BDO and any of the intermediates metabolites in the 4-HB pathway, the BDO pathway and/or the combined 4-HB and BDO pathways. All that is required is to engineer in one or more of the enzyme activities shown in FIG. **1** to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the 4-HB and/or BDO biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that secretes 4-HB when grown on a carbohydrate, secretes BDO when grown on a carbohydrate and/or secretes any of the intermediate metabolites shown in FIG. **1** when grown on a carbohydrate. The BDO producing microbial organisms of the invention can initiate synthesis from, for example, succinate, succinyl-CoA, α-ketogluterate, succinic semialdehyde, 4-HB, 4-hydroxybutyrylphosphate, 4-hydroxybutyryl-CoA (4-HB-CoA) and/or 4-hydroxybutyraldehyde.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described below in the Examples. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the 4-HB and BDO producers can synthesize monomeric 4-HB and BDO, respectively, at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified previously.

A number of downstream compounds also can be generated for the 4-HB and BDO producing non-naturally occurring microbial organisms of the invention. With respect to the 4-HB producing microbial organisms of the invention, monomeric 4-HB and GBL exist in equilibrium in the culture medium. The conversion of 4-HB to GBL can be efficiently accomplished by, for example, culturing the microbial organisms in acid pH medium. A pH less than or equal to 7.5, in particular at or below pH 5.5, spontaneously converts 4-HB to GBL.

The resultant GBL can be separated from 4-HB and other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, the extraction procedures exemplified in the Examples as well as methods which include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art. Separated GBL can be further purified by, for example, distillation.

Another down stream compound that can be produced from the 4-HB producing non-naturally occurring microbial organisms of the invention includes, for example, BDO. This compound can be synthesized by, for example, chemical hydrogenation of GBL. Chemical hydrogenation reactions are well known in the art. One exemplary procedure includes the chemical reduction of 4-HB and/or GBL or a mixture of these two components deriving from the culture using a heterogeneous or homogeneous hydrogenation catalyst together with hydrogen, or a hydride-based reducing agent used stoichiometrically or catalytically, to produce 1,4-butanediol.

Other procedures well known in the art are equally applicable for the above chemical reaction and include, for example, WO No. 82/03854 (Bradley, et al.), which describes the hydrogenolysis of gamma-butyrolactone in the vapor phase over a copper oxide and zinc oxide catalyst. British Pat. No. 1,230,276, which describes the hydrogenation of gamma-butyrolactone using a copper oxide-chromium oxide catalyst. The hydrogenation is carried out in the liquid phase. Batch reactions also are exemplified having high total reactor pressures. Reactant and product partial pressures in the reactors are well above the respective dew points. British Pat. No. 1,314,126, which describes the hydrogenation of gamma-butyrolactone in the liquid phase over a nickel-cobalt-thorium oxide catalyst. Batch reactions are exemplified as having high total pressures and component partial pressures well above respective component dew points. British Pat. No. 1,344,557, which describes the hydrogenation of gamma-butyrolactone in the liquid phase over a copper oxide-chromium oxide catalyst. A vapor phase or vapor-containing mixed phase is indicated as suitable in some instances. A continuous flow tubular reactor is exemplified using high total reactor pressures. British Pat. No. 1,512,751, which describes the hydrogenation of gamma-butyrolactone to 1,4-butanediol in the liquid phase over a copper oxide-chromium oxide catalyst. Batch reactions are exemplified with high total reactor pressures and, where determinable, reactant and product partial pressures well above the respective dew points. U.S. Pat. No. 4,301,077, which describes the hydrogenation to 1,4-butanediol of gamma-butyrolactone over a Ru—Ni—Co—Zn catalyst. The reaction can be conducted in the liquid or gas phase or in a mixed liquid-gas phase. Exemplified are continuous flow liquid phase reactions at high total reactor pressures and relatively low reactor productivities. U.S. Pat. No. 4,048,196, which describes the production of 1,4-butanediol by the liquid phase hydrogenation of gamma-butyrolactone over a copper oxide-zinc oxide catalyst. Further exemplified is a continuous flow tubular reactor operating at high total reactor pressures and high reactant and product partial pressures. And U.S. Pat. No. 4,652,685, which describes the hydrogenation of lactones to glycols.

A further downstream compound that can be produced form the 4-HB producing microbial organisms of the invention includes, for example, THF. This compound can be synthesized by, for example, chemical hydrogenation of GBL. One exemplary procedure well known in the art applicable for the conversion of GBL to THF includes, for example, chemical reduction of 4-HB and/or GBL or a mixture of these two components deriving from the culture using a heterogeneous or homogeneous hydrogenation catalyst together with hydrogen, or a hydride-based reducing agent used stoichiometrically or catalytically, to produce tetrahydrofuran. Other procedures well know in the art are equally applicable for the above chemical reaction and include, for example, U.S. Pat. No. 6,686,310, which describes high surface area sol-gel route prepared hydrogenation catalysts. Processes for the reduction of maleic acid to tetrahydrofuran (THF) and 1,4-butanediol (BDO) and for the reduction of gamma butyrolactone to tetrahydrofuran and 1,4-butanediol also are described.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described further below in the Examples, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

Suitable purification and/or assays to test for the production of 4-HB or BDO can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme or protein activities from the exogenous DNA sequences can also be assayed using methods well known in the art.

The 4-HB or BDO product can be separated from other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art.

The invention further provides a method of manufacturing 4-HB. The method includes fermenting a non-naturally occurring microbial organism having a 4-hydroxybutanoic acid (4-HB) biosynthetic pathway comprising at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, glutamate: succinic semialdehyde transaminase, α-ketoglutarate decarboxylase, or glutamate decarboxylase under substantially anaerobic conditions for a sufficient period of time to produce monomeric 4-hydroxybutanoic acid (4-HB), the process comprising fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation.

The culture and chemical hydrogenations described above also can be scaled up and grown continuously for manufacturing of 4-HB, GBL, BDO and/or THF. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art. Employing the 4-HB producers allows for simultaneous 4-HB biosynthesis and chemical conversion to GBL, BDO and/or THF by employing the above hydrogenation procedures simultaneous with continuous cultures methods such as fermentation. Other hydrogenation procedures also are well known in the art and can be equally applied to the methods of the invention.

Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of 4-HB and/or BDO. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of 4-HB or BDO will include culturing a non-naturally occurring 4-HB or BDO producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. It is further understood that the time of culturing the microbial organism of the invention is for a sufficient period of time to produce a sufficient amount of product for a desired purpose.

Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of 4-HB, BDO or other 4-HB derived products of the invention can be utilized in, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures well known in the art are exemplified further below in the Examples.

In addition, to the above fermentation procedures using the 4-HB or BDO producers of the invention for continuous production of substantial quantities of monomeric 4-HB and BDO, respectively, the 4-HB producers also can be, for example, simultaneously subjected to chemical synthesis procedures as described previously for the chemical conversion of monomeric 4-HB to, for example, GBL, BDO and/or THF. The BDO producers can similarly be, for example, simultaneously subjected to chemical synthesis procedures as described previously for the chemical conversion of BDO to, for example, THF, GBL, pyrrolidones and/or other BDO family compounds. In addition, the products of the 4-HB and BDO producers can be separated from the fermentation culture and sequentially subjected to chemical conversion, as disclosed herein.

Briefly, hydrogenation of GBL in the fermentation broth can be performed as described by Frost et al., *Biotechnology Progress* 18: 201-211 (2002). Another procedure for hydrogenation during fermentation include, for example, the methods described in, for example, U.S. Pat. No. 5,478,952. This method is further exemplified in the Examples below.

Therefore, the invention additionally provides a method of manufacturing α-butyrolactone (GBL), tetrahydrofuran (THF) or 1,4-butanediol (BDO). The method includes fermenting a non-naturally occurring microbial organism having 4-hydroxybutanoic acid (4-HB) and/or 1,4-butanediol (BDO) biosynthetic pathways, the pathways comprise at least one exogenous nucleic acid encoding 4-hydroxybutanoate dehydrogenase, CoA-independent succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, CoA-dependent succinic semialdehyde dehydrogenase, 4-hydroxybutyrate:CoA transferase, glutamate: succinic semialdehyde transaminase, α-ketoglutarate decarboxylase, glutamate decarboxylase, 4-hydroxybutanoate kinase, phosphotransbutyrylase, CoA-independent 1,4-butanediol semialdehyde dehydrogenase, CoA-dependent 1,4-butanediol semialdehyde dehydrogenase, CoA-independent 1,4-butanediol alcohol dehydrogenase or CoA-dependent 1,4-bu-

tanediol alcohol dehydrogenase, under substantially anaerobic conditions for a sufficient period of time to produce 1,4-butanediol (BDO), GBL or THF, the fermenting comprising fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation.

In addition to the biosynthesis of 4-HB, BDO and other products of the invention as described herein, the non-naturally occurring microbial organisms and methods of the invention also can be utilized in various combinations with each other and with other microbial organisms and methods well known in the art to achieve product biosynthesis by other routes. For example, one alternative to produce BDO other than use of the 4-HB producers and chemical steps or other than use of the BDO producer directly is through addition of another microbial organism capable of converting 4-HB or a 4-HB product exemplified herein to BDO.

One such procedure includes, for example, the fermentation of a 4-HB producing microbial organism of the invention to produce 4-HB, as described above and below. The 4-HB can then be used as a substrate for a second microbial organism that converts 4-HB to, for example, BDO, GBL and/or THF. The 4-HB can be added directly to another culture of the second organism or the original culture of 4-HB producers can be depleted of these microbial organisms by, for example, cell separation, and then subsequent addition of the second organism to the fermentation broth can utilized to produce the final product without intermediate purification steps. One exemplary second organism having the capacity to biochemically utilize 4-HB as a substrate for conversion to BDO, for example, is *Clostridium acetobutylicum* (see, for example, Jewell et al., *Current Microbiology,* 13:215-19 (1986)).

In other embodiments, the non-naturally occurring microbial organisms and methods of the invention can be assembled in a wide variety of subpathways to achieve biosynthesis of, for example, 4-HB and/or BDO as described. In these embodiments, biosynthetic pathways for a desired product of the invention can be segregated into different microbial organisms and the different microbial organisms can be co-cultured to produce the final product. In such a biosynthetic scheme, the product of one microbial organism is the substrate for a second microbial organism until the final product is synthesized. For example, the biosynthesis of BDO can be accomplished as described previously by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product, for example, a substrate such as endogenous succinate through 4-HB to the final product BDO. Alternatively, BDO also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel. A first microbial organism being a 4-HB producer with genes to produce 4-HB from succinic acid, and a second microbial organism being a BDO producer with genes to convert 4-HB to BDO.

Given the teachings and guidance provided herein, those skilled in the art will understand that a wide variety of combinations and permutations exist for the non-naturally occurring microbial organisms and methods of the invention together with other microbial organisms, with the co-culture of other non-naturally occurring microbial organisms having subpathways and with combinations of other chemical and/or biochemical procedures well known in the art to produce 4-HB, BDO, GBL and THF products of the invention.

To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Pat. No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of BDO.

One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion or disruption strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed Jan. 10, 2002, in International Patent No. PCT/US02/00660, filed Jan. 10, 2002, and U.S. patent application Ser. No. 11/891,602, filed Aug. 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed Jun. 14, 2002, and in International Patent Application No. PCT/US03/18838, filed Jun. 13, 2003. SimPheny® is a computational system that can be used to produce a network model in silico and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains

any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an in silico method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An in silico stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Pat. No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation,

an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

The methods exemplified above and further illustrated in the Examples below enable the construction of cells and organisms that biosynthetically produce, including obligatory couple production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. In this regard, metabolic alterations have been identified that result in the biosynthesis of 4-HB and 1,4-butanediol. Microorganism strains constructed with the identified metabolic alterations produce elevated levels of 4-HB or BDO compared to unmodified microbial organisms. These strains can be beneficially used for the commercial production of 4-HB, BDO, THF and GBL, for example, in continuous fermentation process without being subjected to the negative selective pressures.

Therefore, the computational methods described herein enable the identification and implementation of metabolic modifications that are identified by an in silico method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the BDO producers can be cultured for the biosynthetic production of BDO.

For the production of BDO, the recombinant strains are cultured in a medium with carbon source and other essential nutrients. It is highly desirable to maintain anaerobic conditions in the fermenter to reduce the cost of the overall process. Such conditions can be obtained, for example, by first sparging the medium with nitrogen and then sealing the flasks with a septum and crimp-cap. For strains where growth is not observed anaerobically, microaerobic conditions can be applied by perforating the septum with a small hole for limited aeration. Exemplary anaerobic conditions have been described previously and are well-known in the art. Exemplary aerobic and anaerobic conditions are described, for example, in U.S. publication 2009/0047719, filed Aug. 10, 2007. Fermentations can be performed in a batch, fed-batch or continuous manner, as disclosed herein.

If desired, the pH of the medium can be maintained at a desired pH, in particular neutral pH, such as a pH of around 7 by addition of a base, such as NaOH or other bases, or acid, as needed to maintain the culture medium at a desirable pH. The growth rate can be determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time.

In addition to renewable feedstocks such as those exemplified above, the BDO producing microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the BDO producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

Synthesis gas, also known as syngas or producer gas, is the major product of gasification of coal and of carbonaceous materials such as biomass materials, including agricultural crops and residues. Syngas is a mixture primarily of H_2 and CO and can be obtained from the gasification of any organic feedstock, including but not limited to coal, coal oil, natural gas, biomass, and waste organic matter. Gasification is generally carried out under a high fuel to oxygen ratio. Although largely H_2 and CO, syngas can also include CO_2 and other gases in smaller quantities. Thus, synthesis gas provides a cost effective source of gaseous carbon such as CO and, additionally, CO_2.

The Wood-Ljungdahl pathway catalyzes the conversion of CO and H_2 to acetyl-CoA and other products such as acetate. Organisms capable of utilizing CO and syngas also generally have the capability of utilizing CO_2 and CO_2/H_2 mixtures through the same basic set of enzymes and transformations encompassed by the Wood-Ljungdahl pathway. H_2-dependent conversion of CO_2 to acetate by microorganisms was recognized long before it was revealed that CO also could be used by the same organisms and that the same pathways were involved. Many acetogens have been shown to grow in the presence of CO_2 and produce compounds such as acetate as long as hydrogen is present to supply the necessary reducing equivalents (see for example, Drake, *Acetogenesis*, pp. 3-60 Chapman and Hall, New York, (1994)). This can be summarized by the following equation:

$$2CO_2+4H_2+n\text{ADP}+n\text{Pi}\rightarrow CH_3COOH+2H_2O+n\text{ATP}$$

Hence, non-naturally occurring microorganisms possessing the Wood-Ljungdahl pathway can utilize CO_2 and H_2 mixtures as well for the production of acetyl-CoA and other desired products.

The Wood-Ljungdahl pathway is well known in the art and consists of 12 reactions which can be separated into two branches: (1) methyl branch and (2) carbonyl branch. The methyl branch converts syngas to methyl-tetrahydrofolate (methyl-THF) whereas the carbonyl branch converts methyl-THF to acetyl-CoA. The reactions in the methyl branch are catalyzed in order by the following enzymes or proteins: ferredoxin oxidoreductase, formate dehydrogenase, formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclodehydratase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase. The reactions in the carbonyl branch are catalyzed in order by the following enzymes or proteins: methyltetrahydrofolate:corrinoid protein methyltransferase (for example, AcsE), corrinoid iron-sulfur protein, nickel-protein assembly protein (for example, AcsF), ferredoxin, acetyl-CoA synthase, carbon monoxide dehydrogenase and nickel-protein assembly protein (for example, CooC). Following the teachings and guidance provided herein for introducing a sufficient number of encoding nucleic acids to generate a BDO pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the Wood-Ljungdahl enzymes or proteins absent in the host organism. Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete Wood-Ljungdahl pathway will confer syngas utilization ability.

Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that

secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, BDO and any of the intermediate metabolites in the BDO pathway. All that is required is to engineer in one or more of the required enzyme or protein activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the BDO biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that produces and/or secretes BDO when grown on a carbohydrate or other carbon source and produces and/or secretes any of the intermediate metabolites shown in the BDO pathway when grown on a carbohydrate or other carbon source. The BDO producing microbial organisms of the invention can initiate synthesis from an intermediate in a BDO pathway, as disclosed herein.

To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Pat. No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of BDO.

One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed Jan. 10, 2002, in International Patent No. PCT/US02/00660, filed Jan. 10, 2002, and U.S. patent application Ser. No. 11/891,602, filed Aug. 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed Jun. 14, 2002, and in International Patent Application No. PCT/US03/18838, filed Jun. 13, 2003. SimPheny® is a computational system that can be used to produce a network model in silico and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through cor-

relation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an in silico method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An in silico stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Pat. No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Biosynthesis of 4-Hydroxybutanoic Acid

This example describes exemplary biochemical pathways for 4-HB production.

Previous reports of 4-HB synthesis in microbes have focused on this compound as an intermediate in production of the biodegradable plastic poly-hydroxyalkanoate (PHA) (U.S. Pat. No. 6,117,658). The use of 4-HB/3-HB copolymers over poly-3-hydroxybutyrate polymer (PHB) can result in plastic that is less brittle (Saito and Doi, *Intl. J. Biol. Macromol.* 16:99-104 (1994)). The production of monomeric 4-HB described herein is a fundamentally distinct process for several reasons: (1) the product is secreted, as opposed to PHA which is produced intracellularly and remains in the cell; (2) for organisms that produce hydroxybutanoate polymers, free 4-HB is not produced, but rather the Coenzyme A derivative is used by the polyhydroxyalkanoate synthase; (3) in the case of the polymer, formation of the granular product changes thermodynamics; and (4) extracellular pH is not an issue for production of the polymer, whereas it will affect whether 4-HB is present in the free acid or conjugate base state, and also the equilibrium between 4-HB and GBL.

4-HB can be produced in two enzymatic reduction steps from succinate, a central metabolite of the TCA cycle, with succinic semialdehyde as the intermediate (FIG. **1**). The first of these enzymes, succinic semialdehyde dehydrogenase, is native to many organisms including *E. coli*, in which both NADH- and NADPH-dependent enzymes have been found (Donnelly and Cooper, *Eur. J. Biochem.* 113:555-561 (1981); Donnelly and Cooper, *J. Bacteriol.* 145:1425-1427 (1981); Marek and Henson, *J. Bacteriol.* 170:991-994 (1988)). There is also evidence supporting succinic semialdehyde dehydrogenase activity in *S. cerevisiae* (Ramos et al., *Eur. J. Biochem.* 149:401-404 (1985)), and a putative

gene has been identified by sequence homology. However, most reports indicate that this enzyme proceeds in the direction of succinate synthesis, as shown in FIG. **1** (Donnelly and Cooper, supra; Lutke-Eversloh and Steinbuchel, *FEMS Microbiol. Lett.* 181:63-71 (1999)), participating in the degradation pathway of 4-HB and gamma-aminobutyrate. Succinic semialdehyde also is natively produced by certain microbial organisms such as *E. coli* through the TCA cycle intermediate α-ketogluterate via the action of two enzymes: glutamate:succinic semialdehyde transaminase and glutamate decarboxylase. An alternative pathway, used by the obligate anaerobe *Clostridium kluyveri* to degrade succinate, activates succinate to succinyl-CoA, then converts succinyl-CoA to succinic semialdehyde using an alternative succinic semialdehyde dehydrogenase which is known to function in this direction (Sohling and Gottschalk, *Eur. J. Biochem.* 212:121-127 (1993)). However, this route has the energetic cost of ATP required to convert succinate to succinyl-CoA.

The second enzyme of the pathway, 4-hydroxybutanoate dehydrogenase, is not native to *E. coli* or yeast but is found in various bacteria such as *C. kluyveri* and *Ralstonia eutropha* (Lutke-Eversloh and Steinbuchel, supra; Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996); Valentin et al., *Eur. J. Biochem.* 227:43-60 (1995); Wolff and Kenealy, *Protein Expr. Purif.* 6:206-212 (1995)). These enzymes are known to be NADH-dependent, though NADPH-dependent forms also exist. An additional pathway to 4-HB from alpha-ketoglutarate was demonstrated in *E. coli* resulting in the accumulation of poly(4-hydroxybutyric acid) (Song et al., *Wei Sheng Wu Xue. Bao.* 45:382-386 (2005)). The recombinant strain required the overexpression of three heterologous genes, PHA synthase (*R. eutropha*), 4-hydroxybutyrate dehydrogenase (*R. eutropha*) and 4-hydroxybutyrate:CoA transferase (*C. kluyveri*), along with two native *E. coli* genes: glutamate:succinic semialdehyde transaminase and glutamate decarboxylase. Steps 4 and 5 in FIG. **1** can alternatively be carried out by an alpha-ketoglutarate decarboxylase such as the one identified in *Euglena gracilis* (Shigeoka et al., *Biochem. J.* 282(Pt2):319-323 (1992); Shigeoka and Nakano, *Arch. Biochem. Biophys.* 288:22-28 (1991); Shigeoka and Nakano, *Biochem J.* 292(Pt 2):463-467 (1993)). However, this enzyme has not previously been applied to impact the production of 4-HB or related polymers in any organism.

The microbial production capabilities of 4-hydroxybutyrate were explored in two microbes, *Escherichia coli* and *Saccharomyces cerevisiae*, using in silico metabolic models of each organism. Potential pathways to 4-HB proceed via a succinate, succinyl-CoA, or alpha-ketoglutarate intermediate as shown in FIG. **1**.

A first step in the 4-HB production pathway from succinate involves the conversion of succinate to succinic semialdehyde via an NADH- or NADPH-dependant succinic semialdehyde dehydrogenase. In *E. coli*, gabD is an NADP-dependant succinic semialdehyde dehydrogenase and is part of a gene cluster involved in 4-aminobutyrate uptake and degradation (Niegemann et al., *Arch. Microbiol.* 160:454-460 (1993); Schneider et al., *J. Bacteriol.* 184:6976-6986 (2002)). sad is believed to encode the enzyme for NAD-dependant succinic semialdehyde dehydrogenase activity (Marek and Henson, supra). *S. cerevisiae* contains only the NADPH-dependant succinic semialdehyde dehydrogenase, putatively assigned to UGA2, which localizes to the cytosol (Huh et al., *Nature* 425:686-691 (2003)). The maximum yield calculations assuming the succinate pathway to 4-HB in both *E. coli* and *S. cerevisiae* require only the assumption that a non-native 4-HB dehydrogenase has been added to their metabolic networks.

The pathway from succinyl-CoA to 4-hydroxybutyrate was described in U.S. Pat. No. 6,117,658 as part of a process for making polyhydroxyalkanoates comprising 4-hydroxybutyrate monomer units. *Clostridium kluyveri* is one example organism known to possess CoA-dependant succinic semialdehyde dehydrogenase activity (Sohling and Gottschalk, supra; Sohling and Gottschalk, supra). In this study, it is assumed that this enzyme, from *C. kluyveri* or another organism, is expressed in *E. coli* or *S. cerevisiae* along with a non-native or heterologous 4-HB dehydrogenase to complete the pathway from succinyl-CoA to 4-HB. The pathway from alpha-ketoglutarate to 4-HB was demonstrated in *E. coli* resulting in the accumulation of poly(4-hydroxybutyric acid) to 30% of dry cell weight (Song et al., supra). As *E. coli* and *S. cerevisiae* natively or endogenously possess both glutamate:succinic semialdehyde transaminase and glutamate decarboxylase (Coleman et al., *J. Biol. Chem.* 276:244-250 (2001)), the pathway from AKG to 4-HB can be completed in both organisms by assuming only that a non-native 4-HB dehydrogenase is present.

EXAMPLE II

Biosynthesis of 1,4-Butanediol from Succinate and Alpha-Ketoglutarate

This example illustrates the construction and biosynthetic production of 4-HB and BDO from microbial organisms. Pathways for 4-HB and BDO are disclosed herein.

There are several alternative enzymes that can be utilized in the pathway described above. The native or endogenous enzyme for conversion of succinate to succinyl-CoA (Step 1 in FIG. **1**) can be replaced by a CoA transferase such as that encoded by the cat1 gene *C. kluyveri* (Sohling and Gottschalk, *Eur. J Biochem.* 212:121-127 (1993)), which functions in a similar manner to Step 9. However, the production of acetate by this enzyme may not be optimal, as it might be secreted rather than being converted back to acetyl-CoA. In this respect, it also can be beneficial to eliminate acetate formation in Step 9. As one alternative to this CoA transferase, a mechanism can be employed in which the 4-HB is first phosphorylated by ATP and then converted to the CoA derivative, similar to the acetate kinase/phosphotransacetylase pathway in *E. coli* for the conversion of acetate to acetyl-CoA. The net cost of this route is one ATP, which is the same as is required to regenerate acetyl-CoA from acetate. The enzymes phosphotransbutyrylase (ptb) and butyrate kinase (bk) are known to carry out these steps on the non-hydroxylated molecules for butyrate production in *C. acetobutylicum* (Cary et al., *Appl Environ Microbiol* 56:1576-1583 (1990); Valentine, R. C. and R. S. Wolfe, *J Biol. Chem.* 235:1948-1952 (1960)). These enzymes are reversible, allowing synthesis to proceed in the direction of 4-HB.

BDO also can be produced via α-ketoglutarate in addition to or instead of through succinate. A described previously, and exemplified further below, one pathway to accomplish product biosynthesis is with the production of succinic semialdehyde via α-ketoglutarate using the endogenous enzymes (FIG. **1**, Steps 4-5). An alternative is to use an α-ketoglutarate decarboxylase that can perform this conversion in one step (FIG. **1**, Step 8; Tian et al., *Proc Natl Acad Sci U.S.A* 102:10670-10675 (2005)).

For the construction of different strains of BDO-producing microbial organisms, a list of applicable genes was assembled for corroboration. Briefly, one or more genes within the 4-HB and/or BDO biosynthetic pathways were identified for each step of the complete BDO-producing pathway shown in FIG. **1**, using available literature resources, the NCBI genetic database, and homology

searches. The genes cloned and assessed in this study are presented below in Table 6, along with the appropriate references and URL citations to the polypeptide sequence. As discussed further below, some genes were synthesized for codon optimization while others were cloned via PCR from the genomic DNA of the native or wild-type organism. For some genes both approaches were used, and in this case the native genes are indicated by an "n" suffix to the gene identification number when used in an experiment. Note that only the DNA sequences differ; the proteins are identical.

TABLE 6

Genes expressed in host BDO-producting microbial organisms.

Gene ID number	Reaction number (FIG. 1)	Gene name	Source organism	Enzyme name	Link to protein sequence	Reference
0001	9	Cat2	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate coenzyme A transferase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0002	12/13	adhE	*Clostridium acetobutylicum* ATCC 824	Aldehyde/alcohol dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=15004739	2
0003	12/13	adhE2	*Clostridium acetobutylicum* ATCC 824	Aldehyde/alcohol dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_149325.1	2
0004	1	Cat1	*Clostridium kluyveri* DSM 555	Succinate coenzyme A transferase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0008	6	sucD	*Clostridium kluyveri* DSM 555	Succinic semialdehyde dehydrogenase (CoA-dependent)	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0009	7	4-HBd	*Ralstonia eutropha* H16	4-hydroxybutyrate dehydrogenase (NAD-dependent)	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=YP_726053.1	2
0010	7	4-HBd	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate dehydrogenase (NAD-dependent)	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1228100	1
0011	12/13	adhE	*E. coli*	Aldehyde/alcohol dehydrogenase	shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do?fromListFlag=true&featureType=1&orfId=1219	
0012	12/13	yqhD	*E. coli*	Aldehyde/alcohol dehydrogenase	shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do	
0013	13	bdhB	*Clostridium acetobutylicum* ATCC 824	Butanol dehydrogenase II	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_349891.1	2
0020	11	ptb	*Clostridium acetobutylicum* ATCC 824	Phospho-transbutyrylase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=15896327	2
0021	10	buk1	*Clostridium acetobutylicum* ATCC 824	Butyrate kinase I	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=20137334	2
0022	10	buk2	*Clostridium acetobutylicum* ATCC 824	Butyrate kinase II	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=20137415	2
0023	13	adhEm	isolated from metalibrary of anaerobic sewage digester microbial consortia	Alcohol dehydrogenase		(37)d}
0024	13	adhE	*Clostridium thermocellum*	Alcohol dehydrogenase	genome.jp/dbget-bin/www_bget?cth:Cthe_0423	
0025	13	ald	*Clostridium beijerinckii*	Coenzyme A-acylating aldehyde dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=49036681	(31)d}
0026	13	bdhA	*Clostridium acetobutylicum* ATCC 824	Butanol dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_349892.1	2
0027	12	bld	*Clostridium saccharoperbutyl-acetonicum*	Butyraldehyde dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=31075383	4
0028	13	bdh	*Clostridium saccharoperbutyl-acetonicum*	Butanol dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=124221917	4
0029	12/13	adhE	*Clostridium tetani*	Aldehyde/alcohol dehydrogenase	genome.jp/dbget-bin/www_bget?ctc:CTC01366	
0030	12/13	adhE	*Clostridium perfringens*	Aldehyde/alcohol dehydrogenase	genome.jp/dbget-bin/www_bget?cpe:CPE2531	
0031	12/13	adhE	*Clostridium difficile*	Aldehyde/alcohol dehydrogenase	genome.jp/dbget-bin/www_bget?cdf:CD2966	

TABLE 6-continued

Genes expressed in host BDO-producting microbial organisms.

Gene ID number	Reaction number (FIG. 1)	Gene name	Source organism	Enzyme name	Link to protein sequence	Reference
0032	8	sucA	*Mycobacterium bovis* BCG, Pasteur	α-ketoglutarate decarboxylase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=YP_977400.1	5
0033	9	cat2	*Clostridium aminobutyricum*	4-hydroxybutyrate coenzyme A transferase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=6249316	
0034	9	cat2	*Porphyromonas gingivalis* W83	4-hydroxybutyrate coenzyme A transferase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=34541558	
0035	6	sucD	*Porphyromonas gingivalis* W83	Succinic semialdehyde dehydrogenase (CoA-dependent)	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_904963.1	
0036	7	4-HBd	*Porphyromonas gingivalis* W83	NAD-dependent 4-hydroxybutyrate dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_904964.1	
0037	7	gbd	Uncultured bacterium	4-hydroxybutyrate dehydrogenase	ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=5916168	6
0038	1	sucCD	*E. coli*	Succinyl-CoA synthetase	shigen.nig.ac.jp/ecoli/pec/genes.List.DetailAction.do	

1 Sohling and Gottschalk, *Eur. J. Biochem.* 212: 121-127 (1993); Sohling and Gottschalk, *J. Bacteriol.* 178: 871-880 (1996)
2 Nolling et al., J., *J. Bacteriol.* 183: 4823-4838 (2001)
3 Pohlmann et al., *Nat. Biotechnol.* 24: 1257-1262 (2006)
4 Kosaka et al., *Biosci. Biotechnol. Biochem.* 71: 58-68 (2007)
5 Brosch et al., *Proc. Natl. Acad. Sci. U.S.A.* 104: 5596-5601 (2007)
6 Henne et al., *Appl. Environ. Microbiol.* 65: 3901-3907 (1999)

Expression Vector Construction for BDO Pathway.

Vector backbones and some strains were obtained from Dr. Rolf Lutz of Expressys (expressys.de/). The vectors and strains are based on the pZ Expression System developed by Dr. Rolf Lutz and Prof. Hermann Bujard (Lutz, R. and H. Bujard, *Nucleic Acids Res* 25:1203-1210 (1997)). Vectors obtained were pZE13luc, pZA33luc, pZS*13luc and pZE22luc and contained the luciferase gene as a stuffer fragment. To replace the luciferase stuffer fragment with a lacZ-alpha fragment flanked by appropriate restriction enzyme sites, the luciferase stuffer fragment was first removed from each vector by digestion with EcoRI and XbaI. The lacZ-alpha fragment was PCR amplified from pUC19 with the following primers:

```
lacZalpha-RI
                                        (SEQ ID NO: 1)
5'GACGAATTCGCTAGCAAGAGGAGAAGTCGACATGTCCAATTCACTGG

CCGTCGTTTTAC3'

lacZalpha 3'BB
                                        (SEQ ID NO: 2)
5'-GACCCTAGGAAGCTTTCTAGAGTCGACCTATGCGGCATCAGAGCAG

A-3'.
```

This generated a fragment with a 5' end of EcoRI site, NheI site, a Ribosomal Binding Site, a SalI site and the start codon. On the 3' end of the fragment contained the stop codon, XbaI, HindIII, and AvrII sites. The PCR product was digested with EcoRI and AvrII and ligated into the base vectors digested with EcoRI and XbaI (XbaI and AvrII have compatible ends and generate a non-site). Because NheI and XbaI restriction enzyme sites generate compatible ends that can be ligated together (but generate a NheI/XbaI non-site that is not digested by either enzyme), the genes cloned into the vectors could be "Biobricked" together (openwetware.org/wiki/Synthetic_Biology:BioBricks). Briefly, this method enables joining an unlimited number of genes into the vector using the same 2 restriction sites (as long as the sites do not appear internal to the genes), because the sites between the genes are destroyed after each addition.

All vectors have the pZ designation followed by letters and numbers indication the origin of replication, antibiotic resistance marker and promoter/regulatory unit. The origin of replication is the second letter and is denoted by E for ColE1, A for p15A and S for pSC101-based origins. The first number represents the antibiotic resistance marker (1 for Ampicillin, 2 for Kanamycin, 3 for Chloramphenicol, 4 for Spectinomycin and 5 for Tetracycline). The final number defines the promoter that regulated the gene of interest (1 for $P_{LtetO-1}$, 2 for $P_{LlacO-1}$, 3 for $P_{A1lacO-1}$, and 4 for $P_{lac/ara-1}$). The MCS and the gene of interest follows immediately after. For the work discussed here we employed two base vectors, pZA33 and pZE13, modified for the biobricks insertions as discussed above. Once the gene(s) of interest have been cloned into them, resulting plasmids are indicated using the four digit gene codes given in Table 6; e.g., pZA33-XXXX-YYYY- . . . .

Host Strain Construction.

The parent strain in all studies described here is *E. coli* K-12 strain MG1655. Markerless deletion strains in adhE, gabD, and aldA were constructed under service contract by a third party using the redET method (Datsenko, K. A. and B. L. Wanner, *Proc Natl Acad Sci U.S.A* 97:6640-6645 (2000)). Subsequent strains were constructed via bacteriophage P1 mediated transduction (Miller, J. Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, New York (1973)). Strain C600Z1 (laci^q, PN25-tetR, Sp^R, lacY1, leuB6, mcrB+, supE44, thi-1, thr-1, tonA21) was obtained from Expressys and was used as a source of a lacI^q allele for P1 transduction. Bacteriophage P1vir was grown on the C600Z1 *E. coli* strain, which has the spectinomycin resistance gene linked to the lacI^q. The P1 lysate grown on

C600Z1 was used to infect MG1655 with selection for spectinomycin resistance. The spectinomycin resistant colonies were then screened for the linked lacI^q by determining the ability of the transductants to repress expression of a gene linked to a $P_{A1lacO-1}$ promoter. The resulting strain was designated MG1655 lacI^q. A similar procedure was used to introduce lacI^Q into the deletion strains.

Production of 4-HB from Succinate.

For construction of a 4-HB producer from succinate, genes encoding steps from succinate to 4-HB and 4-HB-CoA (1, 6, 7, and 9 in FIG. **1**) were assembled onto the pZA33 and pZE13 vectors as described below. Various combinations of genes were assessed, as well as constructs bearing incomplete pathways as controls (Tables 7 and 8). The plasmids were then transformed into host strains containing lacI^Q, which allow inducible expression by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Both wild-type and hosts with deletions in genes encoding the native succinic semialdehyde dehydrogenase (step 2 in FIG. **1**) were tested.

Activity of the heterologous enzymes were first tested in in vitro assays, using strain MG1655 lacI^Q as the host for the plasmid constructs containing the pathway genes. Cells were grown aerobically in LB media (Difco) containing the appropriate antibiotics for each construct, and induced by addition of IPTG at 1 mM when the optical density (OD600) reached approximately 0.5. Cells were harvested after 6 hours, and enzyme assays conducted as discussed below.

In Vitro Enzyme Assays.

To obtain crude extracts for activity assays, cells were harvested by centrifugation at 4,500 rpm (Beckman-Coulter, Allegera X-15R) for 10 min. The pellets were resuspended in 0.3 mL BugBuster (Novagen) reagent with benzonase and lysozyme, and lysis proceeded for 15 minutes at room temperature with gentle shaking. Cell-free lysate was obtained by centrifugation at 14,000 rpm (Eppendorf centrifuge 5402) for 30 min at 4° C. Cell protein in the sample was determined using the method of Bradford et al., *Anal. Biochem.* 72:248-254 (1976), and specific enzyme assays conducted as described below. Activities are reported in Units/mg protein, where a unit of activity is defined as the amount of enzyme required to convert 1 mmol of substrate in 1 min. at room temperature. In general, reported values are averages of at least 3 replicate assays.

Succinyl-CoA transferase (Cat1) activity was determined by monitoring the formation of acetyl-CoA from succinyl-CoA and acetate, following a previously described procedure Sohling and Gottschalk, *J. Bacteriol.* 178:871-880 (1996). Succinyl-CoA synthetase (SucCD) activity was determined by following the formation of succinyl-CoA from succinate and CoA in the presence of ATP. The experiment followed a procedure described by Cha and Parks, *J. Biol. Chem.* 239:1961-1967 (1964). CoA-dependent succinate semialdehyde dehydrogenase (SucD) activity was determined by following the conversion of NAD to NADH at 340 nm in the presence of succinate semialdehyde and CoA (Sohling and Gottschalk, *Eur. J. Biochem.* 212: 121-127 (1993)). 4-HB dehydrogenase (4-HBd) enzyme activity was determined by monitoring the oxidation of NADH to NAD at 340 nm in the presence of succinate semialdehyde. The experiment followed a published procedure Gerhardt et al. *Arch. Microbiol.* 174:189-199 (2000). 4-HB CoA transferase (Cat2) activity was determined using a modified procedure from Scherf and Buckel, *Appl. Environ. Microbiol.* 57:2699-2702 (1991). The formation of 4-HB-CoA or butyryl-CoA formation from acetyl-CoA and 4-HB or butyrate was determined using HPLC.

Alcohol (ADH) and aldehyde (ALD) dehydrogenase was assayed in the reductive direction using a procedure adapted from several literature sources (Durre et al., *FEMS Microbiol. Rev.* 17:251-262 (1995); Palosaari and Rogers, *J. Bacteriol.* 170:2971-2976 (1988) and Welch et al., *Arch. Biochem. Biophys.* 273:309-318 (1989). The oxidation of NADH is followed by reading absorbance at 340 nM every four seconds for a total of 240 seconds at room temperature. The reductive assays were performed in 100 mM MOPS (adjusted to pH 7.5 with KOH), 0.4 mM NADH, and from 1 to 50 μl of cell extract. The reaction is started by adding the following reagents: 100 μl of 100 mM acetaldehyde or butyraldehyde for ADH, or 100 μl of 1 mM acetyl-CoA or butyryl-CoA for ALD. The Spectrophotometer is quickly blanked and then the kinetic read is started. The resulting slope of the reduction in absorbance at 340 nM per minute, along with the molar extinction coefficient of NAD(P)H at 340 nM (6000) and the protein concentration of the extract, can be used to determine the specific activity.

The enzyme activity of PTB is measured in the direction of butyryl-CoA to butyryl-phosphate as described in Cary et al. *J. Bacteriol.* 170:4613-4618 (1988). It provides inorganic phosphate for the conversion, and follows the increase in free CoA with the reagent 5,5'-dithiobis-(2-nitrobenzoic acid), or DTNB. DTNB rapidly reacts with thiol groups such as free CoA to release the yellow-colored 2-nitro-5-mercaptobenzoic acid (TNB), which absorbs at 412 nm with a molar extinction coefficient of 14,140 M cm^{-1}. The assay buffer contained 150 mM potassium phosphate at pH 7.4, 0.1 mM DTNB, and 0.2 mM butyryl-CoA, and the reaction was started by addition of 2 to 50 μL cell extract. The enzyme activity of BK is measured in the direction of butyrate to butyryl-phosphate formation at the expense of ATP. The procedure is similar to the assay for acetate kinase previously described Rose et al., *J. Biol. Chem.* 211:737-756 (1954). However we have found another acetate kinase enzyme assay protocol provided by Sigma to be more useful and sensitive. This assay links conversion of ATP to ADP by acetate kinase to the linked conversion of ADP and phosphoenol pyruvate (PEP) to ATP and pyruvate by pyruvate kinase, followed by the conversion of pyruvate and NADH to lactate and NAD+ by lactate dehydrogenase. Substituting butyrate for acetate is the only major modification to enable the assay to follow BK enzyme activity. The assay mixture contained 80 mM triethanolamine buffer at pH 7.6, 200 mM sodium butyrate, 10 mM $MgCl_2$, 0.1 mM NADH, 6.6 mM ATP, 1.8 mM phosphoenolpyruvate. Pyruvate kinase, lactate dehydrogenase, and myokinase were added according to the manufacturer's instructions. The reaction was started by adding 2 to 50 μL cell extract, and the reaction was monitored based on the decrease in absorbance at 340 nm indicating NADH oxidation.

Analysis of CoA Derivatives by HPLC.

An HPLC based assay was developed to monitor enzymatic reactions involving coenzyme A (CoA) transfer. The developed method enabled enzyme activity characterization by quantitative determination of CoA, acetyl CoA (AcCoA), butyryl CoA (BuCoA) and 4-hydroxybutyrate CoA (4-HB-CoA) present in in-vitro reaction mixtures. Sensitivity down to low μM was achieved, as well as excellent resolution of all the CoA derivatives of interest.

Chemical and sample preparation was performed as follows. Briefly, CoA, AcCoA, BuCoA and all other chemicals, were obtained from Sigma-Aldrich. The solvents, methanol and acetonitrile, were of HPLC grade. Standard calibration curves exhibited excellent linearity in the 0.01-1 mg/mL concentration range. Enzymatic reaction mixtures contained

100 mM Tris HCl buffer (pH 7), aliquots were taken at different time points, quenched with formic acid (0.04% final concentration) and directly analyzed by HPLC.

HPLC analysis was performed using an Agilent 1100 HPLC system equipped with a binary pump, degasser, thermostated autosampler and column compartment, and diode array detector (DAD), was used for the analysis. A reversed phase column, Kromasil 100 5 um C18, 4.6×150 mm (Peeke Scientific), was employed. 25 mM potassium phosphate (pH 7) and methanol or acetonitrile, were used as aqueous and organic solvents at 1 mL/min flow rate. Two methods were developed: a short one with a faster gradient for the analysis of well-resolved CoA, AcCoA and BuCoA, and a longer method for distinguishing between closely eluting AcCoA and 4-HBCoA. Short method employed acetonitrile gradient (0 min-5%, 6 min-30%, 6.5 min-5%, 10 min-5%) and resulted in the retention times 2.7, 4.1 and 5.5 min for CoA, AcCoA and BuCoA, respectively. In the long method methanol was used with the following linear gradient: 0 min-5%, 20 min-35%, 20.5 min-5%, 25 min-5%. The retention times for CoA, AcCoA, 4-HBCoA and BuCoA were 5.8, 8.4, 9.2 and 16.0 min, respectively. The injection volume was 5 μL, column temperature 30° C., and UV absorbance was monitored at 260 nm.

The results demonstrated activity of each of the four pathway steps (Table 7), though activity is clearly dependent on the gene source, position of the gene in the vector, and the context of other genes with which it is expressed. For example, gene 0035 encodes a succinic semialdehyde dehydrogenase that is more active than that encoded by 0008, and 0036 and 0010n are more active 4-HB dehydrogenase genes than 0009. There also seems to be better 4-HB dehydrogenase activity when there is another gene preceding it on the same operon.

TABLE 7

In vitro enzyme activities in cell extracts from MG1655 lacI^{Q} containing the plasmidsexpressing genes in the 4-HB-CoA pathway. Activities are reported in Units/mg protein, where a unit of activity is defined as the amount of enzyme required to convert 1 μmol of substrate in 1 min. at room temperature.

Sample #	pZE13 (a)	pZA33 (b)	OD600	Cell Prot (c)	Cat1	SucD	4HBd	Cat2
1	cat1 (0004)		2.71	6.43	1.232	0.00		
2	cat1 (0004)-sucD (0035)		2.03	5.00	0.761	2.57		
3	cat1 (0004)-sucD (0008)		1.04	3.01	0.783	0.01		
4	sucD (0035)		2.31	6.94		2.32		
5	sucD (0008)		1.10	4.16		0.05		
6		4hbd (0009)	2.81	7.94	0.003		0.25	
7		4hbd (0036)	2.63	7.84			3.31	
8		4hbd (0010n)	2.00	5.08			2.57	
9	cat1 (0004)-sucD (0035)	4hbd (0009)	2.07	5.04	0.600	1.85	0.01	
10	cat1 (0004)-sucD (0035)	4hbd (0036)	2.08	5.40	0.694	1.73	0.41	
11	cat1 (0004)-sucD (0035)	4hbd (0010n)	2.44	4.73	0.679	2.28	0.37	
12	cat1 (0004)-sucD (0008)	4hbd (0009)	1.08	3.99	0.572	−0.01	0.02	
13	cat1 (0004)-sucD (0008)	4hbd (0036)	0.77	2.60	0.898	−0.01	0.04	
14	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.63	2.47	0.776	0.00	0.00	
15		cat2 (0034)	2.56	7.86				1.283
16		cat2(0034)-4hbd(0036)	3.13	8.04			24.86	0.993
17		cat2(0034)-4hbd(0010n)	2.38	7.03			7.45	0.675
18		4hbd(0036)-cat2(0034)	2.69	8.26			2.15	7.490
19		4hbd(0010n)-cat2(0034)	2.44	6.59			0.59	4.101

(a) Genes expressed from Plac on pZE13, a high-copy plasmid with colE1 origin and ampicillin resistance. Gene identification numbers are as given in Table 6

(b) Genes expressed from Plac on pZA33, a medium-copy plasmid with pACYC origin and chloramphenicol resistance.

(c) Cell protein given as mg protein per mL extract.

Recombinant strains containing genes in the 4-HB pathway were then evaluated for the ability to produce 4-HB in vivo from central metabolic intermediates. Cells were grown anaerobically in LB medium to OD600 of approximately 0.4, then induced with 1 mM IPTG. One hour later, sodium succinate was added to 10 mM, and samples taken for analysis following an additional 24 and 48 hours. 4-HB in the culture broth was analyzed by GC-MS as described below. The results indicate that the recombinant strain can produce over 2 mM 4-HB after 24 hours, compared to essentially zero in the control strain (Table 8).

TABLE 8

Production of 4-HB from succinate in *E. coli* strains harboring plasmids expressing various combinations of 4-HB pathway genes.

Sample				24 Hours			48 Hours		
#	Host Strain	pZE13	pZA33	OD600	4HB, μM	4HB norm. (a)	OD600	4HB, μM	4HB norm. (a)
1	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0009)	0.47	487	1036	1.04	1780	1711
2	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0027)	0.41	111	270	0.99	214	217
3	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0036)	0.47	863	1835	0.48	2152	4484
4	MG1655 laclq	cat1 (0004)-sucD (0035)	4hbd (0010n)	0.46	956	2078	0.49	2221	4533

TABLE 8-continued

Production of 4-HB from succinate in *E. coli* strains harboring plasmids expressing various combinations of 4-HB pathway genes.

Sample				24 Hours			48 Hours		
#	Host Strain	pZE13	pZA33	OD600	4HB, μM	4HB norm. (a)	OD600	4HB, μM	4HB norm. (a)
5	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0009)	0.38	493	1296	0.37	1338	3616
6	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0027)	0.32	26	81	0.27	87	323
7	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0036)	0.24	506	2108	0.31	1448	4672
8	MG1655 laclq	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.24	78	324	0.56	233	416
9	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0009)	0.53	656	1237	1.03	1643	1595
10	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0027)	0.44	92	209	0.98	214	218
11	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0036)	0.51	1072	2102	0.97	2358	2431
12	MG1655 laclq gabD	cat1 (0004)-sucD (0035)	4hbd (0010n)	0.51	981	1924	0.97	2121	2186
13	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0009)	0.35	407	1162	0.77	1178	1530
14	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0027)	0.51	19	36	1.07	50	47
15	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0036)	0.35	584	1669	0.78	1350	1731
16	MG1655 laclq gabD	cat1 (0004)-sucD (0008)	4hbd (0010n)	0.32	74	232	0.82	232	283
17	MG1655 laclq	vector only	vector only	0.8	1	2	1.44	3	2
18	MG1655 laclq gabD	vector only	vector only	0.89	1	2	1.41	7	5

(a) Normalized 4-HB concentration, μM/OD600 units

An alternate to using a CoA transferase (cat1) to produce succinyl-CoA from succinate is to use the native *E. coli* sucCD genes, encoding succinyl-CoA synthetase. This gene cluster was cloned onto pZE13 along with candidate genes for the remaining steps to 4-HB to create pZE13-0038-0035-0036.

Production of 4-HB from Glucose.

Although the above experiments demonstrate a functional pathway to 4-HB from a central metabolic intermediate (succinate), an industrial process would require the production of chemicals from low-cost carbohydrate feedstocks such as glucose or sucrose. Thus, the next set of experiments was aimed to determine whether endogenous succinate produced by the cells during growth on glucose could fuel the 4-HB pathway. Cells were grown anaerobically in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. 0.25 mM IPTG was added when OD600 reached approximately 0.2, and samples taken for 4-HB analysis every 24 hours following induction. In all cases 4-HB plateaued after 24 hours, with a maximum of about 1 mM in the best strains (FIG. **3***a*), while the succinate concentration continued to rise (FIG. **3***b*). This indicates that the supply of succinate to the pathway is likely not limiting, and that the bottleneck may be in the activity of the enzymes themselves or in NADH availability. 0035 and 0036 are clearly the best gene candidates for CoA-dependent succinic semialdehyde dehydrogenase and 4-HB dehydrogenase, respectively. The elimination of one or both of the genes encoding known (gabD) or putative (aldA) native succinic semialdehyde dehydrogenases had little effect on performance. Finally, it should be noted that the cells grew to a much lower OD in the 4-HB-producing strains than in the controls (FIG. **3***c*).

An alternate pathway for the production of 4-HB from glucose is via α-ketoglutarate. We explored the use of an α-ketoglutarate decarboxylase from *Mycobacterium tuberculosis* Tian et al., *Proc. Natl. Acad. Sci. USA* 102:10670-10675 (2005) to produce succinic semialdehyde directly from α-ketoglutarate (step 8 in FIG. **1**). To demonstrate that this gene (0032) was functional in vivo, we expressed it on pZE13 in the same host as 4-HB dehydrogenase (gene 0036) on pZA33. This strain was capable of producing over 1.0 mM 4-HB within 24 hours following induction with 1 mM IPTG (FIG. **4**). Since this strain does not express a CoA-dependent succinic semialdehyde dehydrogenase, the possibility of succinic semialdehyde production via succinyl-CoA is eliminated. It is also possible that the native genes responsible for producing succinic semialdehyde could function in this pathway (steps 4 and 5 in FIG. **1**); however, the amount of 4-HB produced when the pZE13-0032 plasmid was left out of the host is the negligible.

Production of BDO from 4-HB.

The production of BDO from 4-HB required two reduction steps, catalyzed by dehydrogenases. Alcohol and aldehyde dehydrogenases (ADH and ALD, respectively) are NAD+/H and/or NADP+/H-dependent enzymes that together can reduce a carboxylic acid group on a molecule to an alcohol group, or in reverse, can perform the oxidation of an alcohol to a carboxylic acid. This biotransformation has been demonstrated in wild-type *Clostridium acetobutylicum* (Jewell et al., *Current Microbiology,* 13:215-19 (1986)), but neither the enzymes responsible nor the genes responsible were identified. In addition, it is not known whether activation to 4-HB-CoA is first required (step 9 in FIG. **1**), or if the aldehyde dehydrogenase (step 12) can act directly on 4-HB. We developed a list of candidate enzymes from *C. acetobutylicum* and related organisms based on known activity with the non-hydroxylated analogues to 4-HB and pathway intermediates, or by similarity to these characterized genes (Table 6). Since some of the candidates are multifunctional dehydrogenases, they could potentially catalyze both the NAD(P)H-dependent reduction of the acid (or CoA-derivative) to the aldehyde, and of the aldehyde to the alcohol. Before beginning work with these genes in *E. coli*, we first validated the result referenced above using *C. acetobutylicum* ATCC 824. Cells were grown in Schaedler broth (Accumedia, Lansing, Mich.) supplemented with 10 mM 4-HB, in an anaerobic atmosphere of 10% CO_2, 10% H_2, and 80% N_2 at 30° C. Periodic culture samples were taken, centrifuged, and the broth analyzed for BDO by GC-MS as described below. BDO concentrations of 0.1 mM, 0.9 mM, and 1.5 mM were detected after 1 day, 2 days, and 7 days incubation, respectively. No BDO was detected in culture grown without 4-HB addition. To demonstrate that the BDO produced was derived from glucose, we grew the best BDO producing strain MG1655 $lacI^Q$ pZE13-0004-0035-0002 pZA33-0034-0036 in M9 minimal medium supplemented with 4 g/L uniformly labeled ^{13}C-glucose. Cells were induced at OD of 0.67 with 1 mM IPTG, and a

sample taken after 24 hours. Analysis of the culture supernatant was performed by mass spectrometry.

Gene candidates for the 4-HB to BDO conversion pathway were next tested for activity when expressed in the *E. coli* host MG1655 lacI^{Q}. Recombinant strains containing each gene candidate expressed on pZA33 were grown in the presence of 0.25 mM IPTG for four hours at 37° C. to fully induce expression of the enzyme. Four hours after induction, cells were harvested and assayed for ADH and ALD activity as described above. Since 4-HB-CoA and 4-hydroxybutyraldehyde are not available commercially, assays were performed using the non-hydroxylated substrates (Table 9). The ratio in activity between 4-carbon and 2-carbon substrates for *C. acetobutylicum* adhE2 (0002) and *E. coli* adhE (0011) were similar to those previously reported in the literature a Atsumi et al., *Biochim. Biophys. Acta.* 1207:1-11 (1994).

TABLE 9

In vitro enzyme activities in cell extracts from MG1655 lacI^{Q} containing pZA33 expressing gene candidates for aldehyde and alcohol dehydrogenases.

	Aldehyde dehydrogenase Substrate		Alcohol dehydrogenase Substrate	
Gene	Butyryl-CoA	Acetyl-CoA	Butyraldehyde	Acetaldehyde
0002	0.0076	0.0046	0.0264	0.0247
0003n	0.0060	0.0072	0.0080	0.0075
0011	0.0069	0.0095	0.0265	0.0093
0013	N.D.	N.D.	0.0130	0.0142
0023	0.0089	0.0137	0.0178	0.0235
0025	0	0.0001	N.D.	N.D.
0026	0	0.0005	0.0024	0.0008

Activities are expressed in μmol min^{-1} mg cell protein^{-1}.
N.D., not determined.

For the BDO production experiments, cat2 from *Porphyromonas gingivalis* W83 (gene 0034) was included on pZA33 for the conversion of 4-HB to 4-HB-CoA, while the candidate dehydrogenase genes were expressed on pZE13. The host strain was MG1655 lacI^{Q}. Along with the alcohol and aldehyde dehydrogenase candidates, we also tested the ability of CoA-dependent succinic semialdehyde dehydrogenases (sucD) to function in this step, due to the similarity of the substrates. Cells were grown to an OD of about 0.5 in LB medium supplemented with 10 mM 4-HB, induced with 1 mM IPTG, and culture broth samples taken after 24 hours and analyzed for BDO as described below. The best BDO production occurred using adhE2 from *C. acetobutylicum*, sucD from *C. kluyveri*, or sucD from *P. gingivalis* (FIG. **5**). Interestingly, the absolute amount of BDO produced was higher under aerobic conditions; however, this is primarily due to the lower cell density achieved in anaerobic cultures. When normalized to cell OD, the BDO production per unit biomass is higher in anaerobic conditions (Table 10).

TABLE 10

Absolute and normalized BDO concentrations from cultures of cells expressing adhE2 from *C. acetobutylicum*, sucD from *C. kluyveri*, or sucD from *P. gingivalis* (data from experiments 2, 9, and 10 in FIG. 3), as well as the negative control (experiment 1).

Gene expressed	Conditions	BDO (μM)	OD (600 nm)	BDO/OD
none	Aerobic	0	13.4	0
none	Microaerobic	0.5	6.7	0.09
none	Anaerobic	2.2	1.26	1.75
0002	Aerobic	138.3	9.12	15.2

TABLE 10-continued

Absolute and normalized BDO concentrations from cultures of cells expressing adhE2 from *C. acetobutylicum*, sucD from *C. kluyveri*, or sucD from *P. gingivalis* (data from experiments 2, 9, and 10 in FIG. 3), as well as the negative control (experiment 1).

Gene expressed	Conditions	BDO (μM)	OD (600 nm)	BDO/OD
0002	Microaerobic	48.2	5.52	8.73
0002	Anaerobic	54.7	1.35	40.5
0008n	Aerobic	255.8	5.37	47.6
0008n	Microaerobic	127.9	3.05	41.9
0008n	Anaerobic	60.8	0.62	98.1
0035	Aerobic	21.3	14.0	1.52
0035	Microaerobic	13.1	4.14	3.16
0035	Anaerobic	21.3	1.06	20.1

As discussed above, it may be advantageous to use a route for converting 4-HB to 4-HB-CoA that does not generate acetate as a byproduct. To this aim, we tested the use of phosphotransbutyrylase (ptb) and butyrate kinase (bk) from *C. acetobutylicum* to carry out this conversion via steps 10 and 11 in FIG. **1**. The native ptb/bk operon from *C. acetobutylicum* (genes 0020 and 0021) was cloned and expressed in pZA33. Extracts from cells containing the resulting construct were taken and assayed for the two enzyme activities as described herein. The specific activity of BK was approximately 65 U/mg, while the specific activity of PTB was approximately 5 U/mg. One unit (U) of activity is defined as conversion of 1 μM substrate in 1 minute at room temperature. Finally, the construct was tested for participation in the conversion of 4-HB to BDO. Host strains were transformed with the pZA33-0020-0021 construct described and pZE13-0002, and compared to use of cat2 in BDO production using the aerobic procedure used above in FIG. **5**. The BK/PTB strain produced 1 mM BDO, compared to 2 mM when using cat2 (Table 11). Interestingly, the results were dependent on whether the host strain contained a deletion in the native adhE gene.

TABLE 11

Absolute and normalized BDO concentrations from cultures of cells expressing adhE2 from *C. acetobutylicum* in pZE13 along with either cat2 from *P. gingivalis* (0034) or the PTB/BK genes from *C. acetobutylicum* on pZA33. Host strains were either MG1655 lacI^{Q} or MG1655 ΔadhE lacI^{Q}.

Genes	Host Strain	BDO (μM)	OD (600 nm)	BDO/OD
0034	MG1655 lacI^{Q}	0.827	19.9	0.042
0020 + 0021	MG1655 lacI^{Q}	0.007	9.8	0.0007
0034	MG1655 ΔadhE lacI^{Q}	2.084	12.5	0.166
0020 + 0021	MG1655 ΔadhE lacI^{Q}	0.975	18.8	0.052

Production of BDO from Glucose.

The final step of pathway corroboration is to express both the 4-HB and BDO segments of the pathway in *E. coli* and demonstrate production of BDO in glucose minimal medium. New plasmids were constructed so that all the required genes fit on two plasmids. In general, cat1, adhE, and sucD genes were expressed from pZE13, and cat2 and 4-HBd were expressed from pZA33. Various combinations of gene source and gene order were tested in the MG1655 lacI^{Q} background. Cells were grown anaerobically in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4C1, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$)

supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. 0.25 mM IPTG was added approximately 15 hours following inoculation, and culture supernatant samples taken for BDO, 4-HB, and succinate analysis 24 and 48 hours following induction. The production of BDO appeared to show a dependency on gene order (Table 12). The highest BDO production, over 0.5 mM, was obtained with cat2 expressed first, followed by 4-HBd on pZA33, and cat1 followed by *P. gingivalis* sucD on pZE13. The addition of *C. acetobutylicum* adhE2 in the last position on pZE13 resulted in slight improvement. 4-HB and succinate were also produced at higher concentrations.

TABLE 12

Production of BDO, 4-HB, and succinate in recombinant *E. coli* strains expressing combinations of BDO pathway genes, grown in minimal medium supplemented with 20 g/L glucose. Concentrations are given in mM.

				24 Hours				48 Hours			
Sample	pZE13	pZA33	Induction OD	OD600 nm	Su	4HB	BDO	OD600 nm	Su	4HB	BDO
1	cat1(0004)-sucD(0035)	4hbd (0036)-cat2(0034)	0.92	1.29	5.44	1.37	0.240	1.24	6.42	1.49	0.280
2	cat1(0004)-sucD(0008N)	4hbd (0036)-cat2(0034)	0.36	1.11	6.90	1.24	0.011	1.06	7.63	1.33	0.011
3	adhE(0002)-cat1(0004)-sucD(0035)	4hbd (0036)-cat2(0034)	0.20	0.44	0.34	1.84	0.050	0.60	1.93	2.67	0.119
4	cat1(0004)-sucD(0035)-adhE(0002)	4hbd (0036)-cat2(0034)	1.31	1.90	9.02	0.73	0.073	1.95	9.73	0.82	0.077
5	adhE(0002)-cat1(0004)-sucD(0008N)	4hbd (0036)-cat2(0034)	0.17	0.45	1.04	1.04	0.008	0.94	7.13	1.02	0.017
6	cat1(0004)-sucD(0008N)-adhE(0002)	4hbd (0036)-cat2(0034)	1.30	1.77	10.47	0.25	0.004	1.80	11.49	0.28	0.003
7	cat1(0004)-sucD(0035)	cat2(0034)-4hbd(0036)	1.09	1.29	5.63	2.15	0.461	1.38	6.66	2.30	0.520
8	cat1(0004)-sucD(0008N)	cat2(0034)-4hbd(0036)	1.81	2.01	11.28	0.02	0.000	2.24	11.13	0.02	0.000
9	adhE(0002)-cat1(0004)-sucD(0035)	cat2(0034)-4hbd(0036)	0.24	1.99	2.02	2.32	0.106	0.89	4.85	2.41	0.186
10	cat1(0004)-sucD(0035)-adhE(0002)	cat2(0034)-4hbd(0036)	0.98	1.17	5.30	2.08	0.569	1.33	6.15	2.14	0.640
11	adhE(0002)-cat1(0004)-sucD(0008N)	cat2(0034)-4hbd(0036)	0.20	0.53	1.38	2.30	0.019	0.91	8.10	1.49	0.034
12	cat1(0004)-sucD(0008N)-adhE(0002)	cat2(0034)-4hbd(0036)	2.14	2.73	12.07	0.16	0.000	3.10	11.79	0.17	0.002
13	vector only	vector only	2.11	2.62	9.03	0.01	0.000	3.00	12.05	0.01	0.000

Analysis of BDO, 4-HB and Succinate by GCMS.

BDO, 4-HB and succinate in fermentation and cell culture samples were derivatized by silylation and quantitatively analyzed by GCMS using methods adapted from literature reports ((Simonov et al., *J. Anal Chem.* 59:965-971 (2004)). The developed method demonstrated good sensitivity down to 1 μM, linearity up to at least 25 mM, as well as excellent selectivity and reproducibility.

Sample preparation was performed as follows: 100 μL filtered (0.2 μm or 0.45 μm syringe filters) samples, e.g. fermentation broth, cell culture or standard solutions, were dried down in a Speed Vac Concentrator (Savant SVC-100H) for approximately 1 hour at ambient temperature, followed by the addition of 20 μL 10 mM cyclohexanol solution, as an internal standard, in dimethylformamide. The mixtures were vortexed and sonicated in a water bath (Branson 3510) for 15 min to ensure homogeneity. 100 μL silylation derivatization reagent, N,O-bis(trimethylsilyl)trifluoro-acetimide (BSTFA) with 1% trimethylchlorosilane, was added, and the mixture was incubated at 70° C. for 30 min. The derivatized samples were centrifuged for 5 min, and the clear solutions were directly injected into GCMS. All the chemicals and reagents were from Sigma-Aldrich, with the exception of BDO which was purchased from J. T. Baker.

GCMS was performed on an Agilent gas chromatograph 6890N, interfaced to a mass-selective detector (MSD) 5973N operated in electron impact ionization (EI) mode has been used for the analysis. A DB-5MS capillary column (J&W Scientific, Agilent Technologies), 30 m×0.25 mm i.d.×0.25 μm film thickness, was used. The GC was operated in a split injection mode introducing 1 μL of sample at 20:1 split ratio. The injection port temperature was 250° C. Helium was used as a carrier gas, and the flow rate was maintained at 1.0 mL/min. A temperature gradient program was optimized to ensure good resolution of the analytes of interest and minimum matrix interference. The oven was initially held at 80° C. for 1 min, then ramped to 120° C. at 2° C./min, followed by fast ramping to 320° C. at 100° C./min and final hold for 6 min at 320° C. The MS interface transfer line was maintained at 280° C. The data were acquired using 'lowmass' MS tune settings and 30-400 m/z mass-range scan. The total analysis time was 29 min including 3 min solvent delay. The retention times corresponded to 5.2, 10.5, 14.0 and 18.2 min for BSTFA-derivatized cyclohexanol, BDO, 4-HB and succinate, respectively. For quantitative analysis, the following specific mass fragments were selected (extracted ion chromatograms): m/z 157 for internal standard cyclohexanol, 116 for BDO, and 147 for both 4-HB and succinate. Standard calibration curves were constructed using analyte solutions in the corresponding cell culture or fermentation medium to match sample matrix as close as possible. GCMS data were processed using Environmental Data Analysis ChemStation software (Agilent Technologies).

The results indicated that most of the 4-HB and BDO produced were labeled with ^{13}C (FIG. **6**, right-hand sides). Mass spectra from a parallel culture grown in unlabeled glucose are shown for comparison (FIG. **6**, left-hand sides). Note that the peaks seen are for fragments of the derivatized molecule containing different numbers of carbon atoms from the metabolite. The derivatization reagent also contributes some carbon and silicon atoms that naturally-occurring label distribution, so the results are not strictly quantitative.

Production of BDO from 4-HB Using Alternate Pathways.

The various alternate pathways were also tested for BDO production. This includes use of the native *E. coli* SucCD enzyme to convert succinate to succinyl-CoA (Table 13, rows 2-3), use of α-ketoglutarate decarboxylase in the α-ketoglutarate pathway (Table 13, row 4), and use of PTB/BK as an alternate means to generate the CoA-derivative of 4HB (Table 13, row 1). Strains were constructed containing plasmids expressing the genes indicated in Table 13, which encompass these variants. The results show that in all cases, production of 4-HB and BDO occurred (Table 13).

TABLE 13

Production of BDO, 4-HB, and succinate in recombinant *E. coli* strains genes for different BDO pathway variants, grown anaerobically in minimal medium supplemented with 20 g/L glucose, and harvested 24 hours after induction with 0.1 mM IPTG. Concentrations are given in mM.

Genes on pZE13	Genes on pZA33	Succinate	4-HB	BDO
0002 + 0004 + 0035	0020n-0021n-0036	0.336	2.91	0.230
0038 + 0035	0034-0036	0.814	2.81	0.126
0038 + 0035	0036-0034	0.741	2.57	0.114
0035 + 0032	0034-0036	5.01	0.538	0.154

EXAMPLE III

Biosynthesis of 4-Hydroxybutanoic Acid, γ-Butyrolactone and 1,4-Butanediol

This Example describes the biosynthetic production of 4-hydroxybutanoic acid, γ-butyrolactone and 1,4-butanediol using fermentation and other bioprocesses.

Methods for the integration of the 4-HB fermentation step into a complete process for the production of purified GBL, 1,4-butanediol (BDO) and tetrahydrofuran (THF) are described below. Since 4-HB and GBL are in equilibrium, the fermentation broth will contain both compounds. At low pH this equilibrium is shifted to favor GBL. Therefore, the fermentation can operate at pH 7.5 or less, generally pH 5.5 or less. After removal of biomass, the product stream enters into a separation step in which GBL is removed and the remaining stream enriched in 4-HB is recycled. Finally, GBL is distilled to remove any impurities. The process operates in one of three ways: 1) fed-batch fermentation and batch separation; 2) fed-batch fermentation and continuous separation; 3) continuous fermentation and continuous separation. The first two of these modes are shown schematically in FIG. **7**. The integrated fermentation procedures described below also are used for the BDO producing cells of the invention for biosynthesis of BDO and subsequent BDO family products.

Fermentation Protocol to Produce 4-HB/GBL (Batch):

The production organism is grown in a 10 L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until 4-HB reaches a concentration of between 20-200 g/L, with the cell density being between 5 and 10 g/L. The pH is not controlled, and will typically decrease to pH 3-6 by the end of the run. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a product separations unit. Isolation of 4-HB and/or GBL would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 4-HB/GBL. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide GBL (boiling point 204-205° C.) which is isolated as a purified liquid.

Fermentation Protocol to Produce 4-HB/GBL (Fully Continuous):

The production organism is first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The 4-HB concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C., and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of 4-HB concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and products 4-HB and/or GBL, is then subjected to a continuous product separations procedure, with or without removing cells and cell debris, and would take place by standard continuous separations methods employed in the art to separate organic products from dilute aqueous solutions, such as continuous liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 4-HB/GBL. The resulting solution is subsequently subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide GBL (boiling point 204-205° C.) which is isolated as a purified liquid.

GBL Reduction Protocol:

Once GBL is isolated and purified as described above, it will then be subjected to reduction protocols such as those well known in the art (references cited) to produce 1,4-butanediol or tetrahydrofuran (THF) or a mixture thereof. Heterogeneous or homogeneous hydrogenation catalysts combined with GBL under hydrogen pressure are well known to provide the products 1,4-butanediol or tetrahydrofuran (THF) or a mixture thereof. It is important to note that the 4-HB/GBL product mixture that is separated from the fermentation broth, as described above, may be subjected directly, prior to GBL isolation and purification, to these same reduction protocols to provide the products 1,4-butanediol or tetrahydrofuran or a mixture thereof. The resulting products, 1,4-butanediol and THF are then isolated and purified by procedures well known in the art.

Fermentation and Hydrogenation Protocol to Produce BDO or THF Directly (Batch):

Cells are grown in a 10 L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until 4-HB reaches a concentration of between 20-200 g/L, with the cell density being between 5 and 10 g/L. The pH is not controlled, and will typically decrease to pH 3-6 by the end of the run. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a reduction unit (e.g., hydrogenation vessel), where the mixture 4-HB/GBL is directly reduced to either 1,4-butanediol or THF or a mixture thereof. Following completion of the reduction procedure, the reactor contents are transferred to a product separations

unit. Isolation of 1,4-butanediol and/or THF would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 1,4-butanediol and/or THF. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide 1,4-butanediol and/or THF which are isolated as a purified liquids.

Fermentation and Hydrogenation Protocol to Produce BDO or THF Directly (Fully Continuous):

The cells are first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The 4-HB concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C., and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of 4-HB concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and products 4-HB and/or GBL, is then passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a continuous reduction unit (e.g., hydrogenation vessel), where the mixture 4-HB/GBL is directly reduced to either 1,4-butanediol or THF or a mixture thereof. Following completion of the reduction procedure, the reactor contents are transferred to a continuous product separations unit. Isolation of 1,4-butanediol and/or THF would take place by standard continuous separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of 1,4-butanediol and/or THF. The resulting solution is then subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide 1,4-butanediol and/or THF which are isolated as a purified liquids.

Fermentation Protocol to Produce BDO Directly (Batch):

The production organism is grown in a 10 L bioreactor sparged with an N_2/CO_2 mixture, using 5 L broth containing 5 g/L potassium phosphate, 2.5 g/L ammonium chloride, 0.5 g/L magnesium sulfate, and 30 g/L corn steep liquor, and an initial glucose concentration of 20 g/L. As the cells grow and utilize the glucose, additional 70% glucose is fed into the bioreactor at a rate approximately balancing glucose consumption. The temperature of the bioreactor is maintained at 30 degrees C. Growth continues for approximately 24 hours, until BDO reaches a concentration of between 20-200 g/L, with the cell density generally being between 5 and 10 g/L. Upon completion of the cultivation period, the fermenter contents are passed through a cell separation unit (e.g., centrifuge) to remove cells and cell debris, and the fermentation broth is transferred to a product separations unit. Isolation of BDO would take place by standard separations procedures employed in the art to separate organic products from dilute aqueous solutions, such as liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of BDO. The resulting solution is then subjected to standard distillation methods to remove and recycle the organic solvent and to provide BDO (boiling point 228-229° C.) which is isolated as a purified liquid.

Fermentation Protocol to Produce BDO Directly (Fully Continuous):

The production organism is first grown up in batch mode using the apparatus and medium composition described above, except that the initial glucose concentration is 30-50 g/L. When glucose is exhausted, feed medium of the same composition is supplied continuously at a rate between 0.5 L/hr and 1 L/hr, and liquid is withdrawn at the same rate. The BDO concentration in the bioreactor remains constant at 30-40 g/L, and the cell density remains constant between 3-5 g/L. Temperature is maintained at 30 degrees C., and the pH is maintained at 4.5 using concentrated NaOH and HCl, as required. The bioreactor is operated continuously for one month, with samples taken every day to assure consistency of BDO concentration. In continuous mode, fermenter contents are constantly removed as new feed medium is supplied. The exit stream, containing cells, medium, and the product BDO, is then subjected to a continuous product separations procedure, with or without removing cells and cell debris, and would take place by standard continuous separations methods employed in the art to separate organic products from dilute aqueous solutions, such as continuous liquid-liquid extraction using a water immiscible organic solvent (e.g., toluene) to provide an organic solution of BDO. The resulting solution is subsequently subjected to standard continuous distillation methods to remove and recycle the organic solvent and to provide BDO (boiling point 228-229° C.) which is isolated as a purified liquid (mpt 20° C.).

EXAMPLE IV

Exemplary BDO Pathways

This example describes exemplary enzymes and corresponding genes for 1,4-butandiol (BDO) synthetic pathways.

Exemplary BDO synthetic pathways are shown in FIGS. **8-13**. The pathways depicted in FIGS. **8-13** are from common central metabolic intermediates to 1,4-butanediol. All transformations depicted in FIGS. **8-13** fall into the 18 general categories of transformations shown in Table 14. Below is described a number of biochemically characterized candidate genes in each category. Specifically listed are genes that can be applied to catalyze the appropriate transformations in FIGS. **9-13** when cloned and expressed in a host organism. The top three exemplary genes for each of the key steps in FIGS. **9-13** are provided in Tables 15-23 (see below). Exemplary genes were provided for the pathways depicted in FIG. **8** are described herein.

TABLE 14

Enzyme types required to convert common central metabolic intermediates into 1,4-butanediol. The first three digits of each label correspond to the first three Enzyme Commission number digits which denote the general type of transformation independent of substrate specificity.

Label	Function
1.1.1.a	Oxidoreductase (ketone to hydroxyl or aldehyde to alcohol)
1.1.1.c	Oxidoreductase (2 step, acyl-CoA to alcohol)
1.2.1.b	Oxidoreductase (acyl-CoA to aldehyde)
1.2.1.c	Oxidoreductase (2-oxo acid to acyl-CoA, decarboxylation)

TABLE 14-continued

Enzyme types required to convert common central metabolic intermediates into 1,4-butanediol. The first three digits of each label correspond to the first three Enzyme Commission number digits which denote the general type of transformation independent of substrate specificity.

Label	Function
1.2.1.d	Oxidoreductase (phosphorylating/dephosphorylating)
1.3.1.a	Oxidoreductase operating on CH—CH donors
1.4.1.a	Oxidoreductase operating on amino acids
2.3.1.a	Acyltransferase (transferring phosphate group)
2.6.1.a	Aminotransferase
2.7.2.a	Phosphotransferase, carboxyl group acceptor
2.8.3.a	Coenzyme-A transferase
3.1.2.a	Thiolester hydrolase (CoA specific)
4.1.1.a	Carboxy-lyase
4.2.1.a	Hydro-lyase
4.3.1.a	Ammonia-lyase
5.3.3.a	Isomerase
5.4.3.a	Aminomutase
6.2.1.a	Acid-thiol ligase

1.1.1.a—Oxidoreductase (Aldehyde to Alcohol or Ketone to Hydroxyl)

Aldehyde to Alcohol.

Exemplary genes encoding enzymes that catalyze the conversion of an aldehyde to alcohol, that is, alcohol dehydrogenase or equivalently aldehyde reductase, include alrA encoding a medium-chain alcohol dehydrogenase for C2-C14 (Tani et al. *Appl. Environ. Microbiol.* 66:5231-5235 (2000)), ADH2 from *Saccharomyces cerevisiae* (Atsumi et al. *Nature* 451:86-89 (2008)), yqhD from *E. coli* which has preference for molecules longer than C(3) (Sulzenbacher et al. *Journal of Molecular Biology* 342:489-502 (2004)), and bdh I and bdh II from *C. acetobutylicum* which converts butyraldehyde into butanol (Walter et al. *Journal of Bacteriology* 174:7149-7158 (1992)). The protein sequences for each of these exemplary gene products, if available, can be found using the following GenBank accession numbers:

alrA	BAB12273.1	*Acinetobacter* sp. Strain M-1
ADH2	NP_014032.1	*Saccharymyces cerevisiae*
yqhD	NP_417484.1	*Escherichia coli*
bdh I	NP_349892.1	*Clostridium acetobutylicum*
bdh II	NP_349891.1	*Clostridium acetobutylicum*

Enzymes exhibiting 4-hydroxybutyrate dehydrogenase activity (EC 1.1.1.61) also fall into this category. Such enzymes have been characterized in *Ralstonia eutropha* (Bravo et al. *J. Forensic Sci.* 49:379-387 (2004)), *Clostridium kluyveri* (Wolff et al. *Protein Expr. Purif.* 6:206-212 (1995)) and *Arabidopsis thaliana* (Breitkreuz et al. *J. Biol. Chem.* 278:41552-41556 (2003)).

4hbd	YP_726053.1	*Ralstonia eutropha* H16
4hbd	L21902.1	*Clostridium kluyveri* DSM 555
4hbd	Q94B07	*Arabidopsis thaliana*

Another exemplary enzyme is 3-hydroxyisobutyrate dehydrogenase which catalyzes the reversible oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde. This enzyme participates in valine, leucine and isoleucine degradation and has been identified in bacteria, eukaryotes, and mammals. The enzyme encoded by P84067 from *Thermus thermophilus* HB8 has been structurally characterized (Lokanath et al. *J Mol Biol* 352:905-17 (2005)). The reversibility of the human 3-hydroxyisobutyrate dehydrogenase was demonstrated using isotopically-labeled substrate (Manning et al. *Biochem J* 231:481-484 (1985)). Additional genes encoding this enzyme include 3hidh in *Homo sapiens* (Hawes et al. *Methods Enzymol.* 324:218-228 (2000)) and *Oryctolagus cuniculus* (Chowdhury et al. *Biosci. Biotechnol Biochem.* 60:2043-2047 (1996); Hawes et al. *Methods Enzymol.* 324:218-228 (2000)), mmsb in *Pseudomonas aeruginosa*, and dhat in *Pseudomonas putida* (Aberhart et al. *J Chem. Soc. [Perkin* 1] 6:1404-1406 (1979); Chowdhury et al. *Biosci. Biotechnol Biochem.* 67:438-441 (2003); Chowdhury et al. *Biosci. Biotechnol Biochem.* 60:2043-2047 (1996)).

P84067	P84067	*Thermus thermophilus*
mmsb	P28811.1	*Pseudomonas aeruginosa*
dhat	Q59477.1	*Pseudomonas putida*
3hidh	P31937.2	*Homo sapiens*
3hidh	P32185.1	*Oryctolagus cuniculus*

Several 3-hydroxyisobutyrate dehydrogenase enzymes have also been shown to convert malonic semialdehyde to 3-hydroxypropionic acid (3-HP). Three gene candidates exhibiting this activity are mmsB from *Pseudomonas aeruginosa* PAO1(62), mmsB from *Pseudomonas putida* KT2440 (Liao et al., US Publication 2005/0221466) and mmsB from *Pseudomonas putida* E23 (Chowdhury et al., *Biosci. Biotechnol. Biochem.* 60:2043-2047 (1996)). An enzyme with 3-hydroxybutyrate dehydrogenase activity in *Alcaligenes faecalis* M3A has also been identified (Gokam et al., U.S. Pat. No. 7,393,676; Liao et al., US Publication No. 2005/0221466). Additional gene candidates from other organisms including *Rhodobacter spaeroides* can be inferred by sequence similarity.

mmsB	AAA25892.1	*Pseudomonas aeruginosa*
mmsB	NP_252259.1	*Pseudomonas aeruginosa* PAO1
mmsB	NP_746775.1	*Pseudomonas putida* KT2440
mmsB	JC7926	*Pseudomonas putida* E23
orfB1	AAL26884	*Rhodobacter spaeroides*

The conversion of malonic semialdehyde to 3-HP can also be accomplished by two other enzymes: NADH-dependent 3-hydroxypropionate dehydrogenase and NADPH-dependent malonate semialdehyde reductase. An NADH-dependent 3-hydroxypropionate dehydrogenase is thought to participate in beta-alanine biosynthesis pathways from propionate in bacteria and plants (Rathinasabapathi, B. *Journal of Plant Pathology* 159:671-674 (2002); Stadtman, E. R. *J. Am. Chem. Soc.* 77:5765-5766 (1955)). This enzyme has not been associated with a gene in any organism to date. NADPH-dependent malonate semialdehyde reductase catalyzes the reverse reaction in autotrophic CO_2-fixing bacteria. Although the enzyme activity has been detected in *Metallosphaera sedula*, the identity of the gene is not known (Alber et al. *J. Bacteriol.* 188:8551-8559 (2006)).

Ketone to Hydroxyl.

There exist several exemplary alcohol dehydrogenases that convert a ketone to a hydroxyl functional group. Two such enzymes from *E. coli* are encoded by malate dehydrogenase (mdh) and lactate dehydrogenase (ldhA). In addition, lactate dehydrogenase from *Ralstonia eutropha* has been shown to demonstrate high activities on substrates of various chain lengths such as lactate, 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (Steinbuchel, A. and H. G. Schlegel *Eur. J. Biochem.* 130:329-334 (1983)). Conversion of alpha-

ketoadipate into alpha-hydroxyadipate can be catalyzed by 2-ketoadipate reductase, an enzyme reported to be found in rat and in human placenta (Suda et al. Arch. Biochem. Biophys. 176:610-620 (1976); Suda et al. *Biochem. Biophys. Res. Commun.* 77:586-591 (1977)). An additional candidate for this step is the mitochondrial 3-hydroxybutyrate dehydrogenase (bdh) from the human heart which has been cloned and characterized (Marks et al. *J. Biol. Chem.* 267:15459-15463 (1992)). This enzyme is a dehydrogenase that operates on a 3-hydroxyacid. Another exemplary alcohol dehydrogenase converts acetone to isopropanol as was shown in *C. beijerinckii* (Ismaiel et al. *J. Bacteriol.* 175: 5097-5105 (1993)) and *T. brockii* (Lamed et al. *Biochem. J.* 195:183-190 (1981); Peretz and Burstein *Biochemistry* 28:6549-6555 (1989)).

mdh	AAC76268.1	*Escherichia coli*
ldhA	NP_415898.1	*Escherichia coli*
ldh	YP_725182.1	*Ralstonia eutropha*
bdh	AAA58352.1	*Homo sapiens*
adh	AAA23199.2	*Clostridium beijerinckii* NRRL B593
adh	P14941.1	*Thermoanaerobacter brockii* HTD4

Exemplary 3-hydroxyacyl dehydrogenases which convert acetoacetyl-CoA to 3-hydroxybutyryl-CoA include hbd from *C. acetobutylicum* (Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996)), hbd from *C. beijerinckii* (Colby et al. *Appl Environ. Microbiol* 58:3297-3302 (1992)), and a number of similar enzymes from *Metallosphaera sedula* (Berg et al. Archaea. Science. 318:1782-1786 (2007)).

hbd	NP_349314.1	*Clostridium acetobutylicum*
hbd	AAM14586.1	*Clostridium beijerinckii*
Msed_1423	YP_001191505	*Metallosphaera sedula*
Msed_0399	YP_001190500	*Metallosphaera sedula*
Msed_0389	YP_001190490	*Metallosphaera sedula*
Msed_1993	YP_001192057	*Metallosphaera sedula*

1.1.1.c—Oxidoredutase (2 Step, acyl-CoA to Alcohol)

Exemplary 2-step oxidoreductases that convert an acyl-CoA to alcohol include those that transform substrates such as acetyl-CoA to ethanol (for example, adhE from *E. coli* (Kessler et al. *FEBS. Lett.* 281:59-63 (1991)) and butyryl-CoA to butanol (for example, adhE2 from *C. acetobutylicum* (Fontaine et al. *J. Bacteriol.* 184:821-830 (2002)). In addition to reducing acetyl-CoA to ethanol, the enzyme encoded by adhE in *Leuconostoc mesenteroides* has been shown to oxide the branched chain compound isobutyraldehyde to isobutyryl-CoA (Kazahaya et al. *J. Gen. Appl. Microbiol.* 18:43-55 (1972); Koo et al. Biotechnol Lett. 27:505-510 (2005)).

adhE	NP_415757.1	*Escherichia coli*
adhE2	AAK09379.1	*Clostridium acetobutylicum*
adhE	AAV66076.1	*Leuconostoc mesenteroides*

Another exemplary enzyme can convert malonyl-CoA to 3-HP. An NADPH-dependent enzyme with this activity has characterized in *Chloroflexus aurantiacus* where it participates in the 3-hydroxypropionate cycle (Hugler et al., *J. Bacteriol.* 184:2404-2410 (2002); Strauss and Fuchs, *Eur. J. Biochem.* 215:633-643 (1993)). This enzyme, with a mass of 300 kDa, is highly substrate-specific and shows little sequence similarity to other known oxidoreductases (Hugler et al., *J. Bacteriol.* 184:2404-2410 (2002)). No enzymes in other organisms have been shown to catalyze this specific reaction; however there is bioinformatic evidence that other organisms may have similar pathways (Klatt et al., *Environ. Microbiol.* 9:2067-2078 (2007)). Enzyme candidates in other organisms including *Roseiflexus castenholzii, Erythrobacter* sp. NAP1 and marine gamma proteobacterium HTCC2080 can be inferred by sequence similarity.

mcr	AAS20429.1	*Chloroflexus aurantiacus*
Rcas_2929	YP_001433009.1	*Roseiflexus castenholzii*
NAP1_02720	ZP_01039179.1	*Erythrobacter* sp. NAP1
MGP2080_00535	ZP_01626393.1	marine gamma proteobacterium HTCC2080

Longer chain acyl-CoA molecules can be reduced by enzymes such as the jojoba (*Simmondsia chinensis*) FAR which encodes an alcohol-forming fatty acyl-CoA reductase. Its overexpression in *E. coli* resulted in FAR activity and the accumulation of fatty alcohol (Metz et al. *Plant Physiology* 122:635-644) 2000)).

FAR	AAD38039.1	*Simmondsia chinensis*

1.2.1.b—Oxidoreductase (acyl-CoA to Aldehyde)

Several acyl-CoA dehydrogenases are capable of reducing an acyl-CoA to its corresponding aldehyde. Exemplary genes that encode such enzymes include the *Acinetobacter calcoaceticus* acyl encoding a fatty acyl-CoA reductase (Reiser and Somerville, *J. Bacteriology* 179:2969-2975 (1997)), the *Acinetobacter* sp. M-1 fatty acyl-CoA reductase (Ishige et al. *Appl. Environ. Microbiol.* 68:1192-1195 (2002)), and a CoA- and NADP-dependent succinate semialdehyde dehydrogenase encoded by the sucD gene in *Clostridium kluyveri* (Sohling and Gottschalk *J Bacteriol* 178:871-80 (1996); Sohling and Gottschalk J. Bacteriol. 178:871-880 (1996)). SucD of *P. gingivalis* is another succinate semialdehyde dehydrogenase (Takahashi et al. *J. Bacteriol.* 182:4704-4710 (2000)). The enzyme acylating acetaldehyde dehydrogenase in *Pseudomonas* sp, encoded by bphG, is yet another as it has been demonstrated to oxidize and acylate acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde and formaldehyde (Powlowski et al. *J Bacteriol.* 175:377-385 (1993)).

acr1	YP_047869.1	*Acinetobacter calcoaceticus*
acr1	AAC45217	*Acinetobacter baylyi*
acr1	BAB85476.1	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	*Clostridium kluyveri*
sucD	NP_904963.1	*Porphyromonas gingivalis*
bphG	BAA03892.1	*Pseudomonas* sp

An additional enzyme type that converts an acyl-CoA to its corresponding aldehyde is malonyl-CoA reductase which transforms malonyl-CoA to malonic semialdehyde. Malonyl-CoA reductase is a key enzyme in autotrophic carbon fixation via the 3-hydroxypropionate cycle in thermoacidophilic archael bacteria (Berg et al. *Science* 318:1782-1786 (2007); Thauer, R. K. *Science* 318:1732-1733 (2007)). The enzyme utilizes NADPH as a cofactor and has been characterized in *Metallosphaera* and *Sulfolobus* spp (Alber et al. *J. Bacteriol.* 188:8551-8559 (2006); Hugler et al. *J. Bacteriol.* 184:2404-2410 (2002)). The enzyme is encoded by Msed_0709 in *Metallosphaera sedula* (Alber et al. *J. Bacteriol.* 188:8551-8559 (2006); Berg et al. *Science* 318:1782-

1786 (2007)). A gene encoding a malonyl-CoA reductase from *Sulfolobus tokodaii* was cloned and heterologously expressed in *E. coli* (Alber et al. *J. Bacteriol.* 188:8551-8559 (2006)). Although the aldehyde dehydrogenase functionality of these enzymes is similar to the bifunctional dehydrogenase from *Chloroflexus aurantiacus*, there is little sequence similarity. Both malonyl-CoA reductase enzyme candidates have high sequence similarity to aspartate-semialdehyde dehydrogenase, an enzyme catalyzing the reduction and concurrent dephosphorylation of aspartyl-4-phosphate to aspartate semialdehyde. Additional gene candidates can be found by sequence homology to proteins in other organisms including *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*.

Msed_0709	YP_001190808.1	*Metallosphaera sedula*
mcr	NP_378167.1	*Sulfolobus tokodaii*
asd-2	NP_343563.1	*Sulfolobus solfataricus*
Saci_2370	YP_256941.1	*Sulfolobus acidocaldarius*

1.2.1.c—Oxidoreductase (2-oxo Acid to acyl-CoA, Decarboxylation)

Enzymes in this family include 1) branched-chain 2-keto-acid dehydrogenase, 2) alpha-ketoglutarate dehydrogenase, and 3) the pyruvate dehydrogenase multienzyme complex (PDHC). These enzymes are multi-enzyme complexes that catalyze a series of partial reactions which result in acylating oxidative decarboxylation of 2-keto-acids. Each of the 2-keto-acid dehydrogenase complexes occupies key positions in intermediary metabolism, and enzyme activity is typically tightly regulated (Fries et al. *Biochemistry* 42:6996-7002 (2003)). The enzymes share a complex but common structure composed of multiple copies of three catalytic components: alpha-ketoacid decarboxylase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). The E3 component is shared among all 2-keto-acid dehydrogenase complexes in an organism, while the E1 and E2 components are encoded by different genes. The enzyme components are present in numerous copies in the complex and utilize multiple cofactors to catalyze a directed sequence of reactions via substrate channeling. The overall size of these dehydrogenase complexes is very large, with molecular masses between 4 and 10 million Da (that is, larger than a ribosome).

Activity of enzymes in the 2-keto-acid dehydrogenase family is normally low or limited under anaerobic conditions in *E. coli*. Increased production of NADH (or NADPH) could lead to a redox-imbalance, and NADH itself serves as an inhibitor to enzyme function. Engineering efforts have increased the anaerobic activity of the *E. coli* pyruvate dehydrogenase complex (Kim et al. *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim et al. *J. Bacteriol.* 190: 3851-3858) 2008); Zhou et al. *Biotechnol. Lett.* 30:335-342 (2008)). For example, the inhibitory effect of NADH can be overcome by engineering an H322Y mutation in the E3 component (Kim et al. *J. Bacteriol.* 190:3851-3858 (2008)). Structural studies of individual components and how they work together in complex provide insight into the catalytic mechanisms and architecture of enzymes in this family (Aevarsson et al. *Nat. Struct. Biol.* 6:785-792 (1999); Zhou et al. *Proc. Natl. Acad. Sci. U.S.A.* 98:14802-14807 (2001)). The substrate specificity of the dehydrogenase complexes varies in different organisms, but generally branched-chain keto-acid dehydrogenases have the broadest substrate range.

Alpha-ketoglutarate dehydrogenase (AKGD) converts alpha-ketoglutarate to succinyl-CoA and is the primary site of control of metabolic flux through the TCA cycle (Hansford, R. G. *Curr. Top. Bioenerg.* 10:217-278 (1980)). Encoded by genes sucA, sucB and lpd in *E. coli*, AKGD gene expression is downregulated under anaerobic conditions and during growth on glucose (Park et al. *Mol. Microbiol.* 15:473-482 (1995)). Although the substrate range of AKGD is narrow, structural studies of the catalytic core of the E2 component pinpoint specific residues responsible for substrate specificity (Knapp et al. *J. Mol. Biol.* 280:655-668 (1998)). The *Bacillus subtilis* AKGD, encoded by odhAB (E1 and E2) and pdhD (E3, shared domain), is regulated at the transcriptional level and is dependent on the carbon source and growth phase of the organism (Resnekov et al. *Mol. Gen. Genet.* 234:285-296 (1992)). In yeast, the LPD1 gene encoding the E3 component is regulated at the transcriptional level by glucose (Roy and Dawes *J. Gen. Microbiol.* 133:925-933 (1987)). The E1 component, encoded by KGDJ, is also regulated by glucose and activated by the products of HAP2 and HAP3 (Repetto and Tzagoloff *Mol. Cell Biol.* 9:2695-2705 (1989)). The AKGD enzyme complex, inhibited by products NADH and succinyl-CoA, is well-studied in mammalian systems, as impaired function of has been linked to several neurological diseases (Tretter and dam-Vizi *Philos. Trans. R. Soc. Lond B Biol. Sci.* 360:2335-2345 (2005)).

sucA	NP_415254.1	*Escherichia coli* str. K12 substr. MG1655
sucB	NP_415255.1	*Escherichia coli* str. K12 substr. MG1655
lpd	NP_414658.1	*Escherichia coli* str. K12 substr. MG1655
odhA	P23129.2	*Bacillus subtilis*
odhB	P16263.1	*Bacillus subtilis*
pdhD	P21880.1	*Bacillus subtilis*
KGD1	NP_012141.1	*Saccharomyces cerevisiae*
KGD2	NP_010432.1	*Saccharomyces cerevisiae*
LPD1	NP_116635.1	*Saccharomyces cerevisiae*

Branched-chain 2-keto-acid dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase, participates in branched-chain amino acid degradation pathways, converting 2-keto acids derivatives of valine, leucine and isoleucine to their acyl-CoA derivatives and CO_2. The complex has been studied in many organisms including *Bacillus subtilis* (Wang et al. *Eur. J. Biochem.* 213:1091-1099 (1993)), *Rattus norvegicus* (Namba et al. *J. Biol. Chem.* 244:4437-4447 (1969)) and *Pseudomonas putida* (Sokatch *J. Bacteriol.* 148:647-652 (1981)). In *Bacillus subtilis* the enzyme is encoded by genes pdhD (E3 component), bfmBB (E2 component), bfmBAA and bfmBAB (E1 component) (Wang et al. *Eur. J. Biochem.* 213:1091-1099 (1993)). In mammals, the complex is regulated by phosphorylation by specific phosphatases and protein kinases. The complex has been studied in rat hepatocites (Chicco et al. *J. Biol. Chem.* 269:19427-19434 (1994)) and is encoded by genes Bckdha (E1 alpha), Bckdhb (E1 beta), Dbt (E2), and Dld (E3). The E1 and E3 components of the *Pseudomonas putida* BCKAD complex have been crystallized (Aevarsson et al. *Nat. Struct. Biol.* 6:785-792 (1999); Mattevi *Science* 255:1544-1550 (1992)) and the enzyme complex has been studied (Sokatch et al. *J. Bacteriol.* 148:647-652 (1981)). Transcription of the *P. putida* BCKAD genes is activated by the gene product of bkdR (Hester et al. *Eur. J. Biochem.* 233:828-836 (1995)). In some organisms including *Rattus norvegicus* (Paxton et al. *Biochem. J.* 234:295-303 (1986)) and *Saccharomyces cerevisiae* (Sinclair et al. *Biochem. Mol. Biol. Int.* 31:911-922 (1993)), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanoate and alpha-ketoglutarate, in addition

to the branched-chain amino acid precursors. The active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, *Biochemistry* 33:12879-12885 (1994)).

bfmBB	NP_390283.1	*Bacillus subtilis*
bfmBAA	NP_390285.1	*Bacillus subtilis*
bfmBAB	NP_390284.1	*Bacillus subtilis*
pdhD	P21880.1	*Bacillus subtilis*
lpdV	P09063.1	*Pseudomonas putida*
bkdB	P09062.1	*Pseudomonas putida*
bkdA1	NP_746515.1	*Pseudomonas putida*
bkdA2	NP_746516.1	*Pseudomonas putida*
Bckdha	NP_036914.1	*Rattus norvegicus*
Bckdhb	NP_062140.1	*Rattus norvegicus*
Dbt	NP_445764.1	*Rattus norvegicus*
Dld	NP_955417.1	*Rattus norvegicus*

The pyruvate dehydrogenase complex, catalyzing the conversion of pyruvate to acetyl-CoA, has also been extensively studied. In the *E. coli* enzyme, specific residues in the E1 component are responsible for substrate specificity (Bisswanger, H. *J Biol. Chem.* 256:815-822 (1981); Bremer, J. *Eur. J Biochem.* 8:535-540 (1969); Gong et al. *J Biol. Chem.* 275:13645-13653 (2000)). As mentioned previously, enzyme engineering efforts have improved the *E. coli* PDH enzyme activity under anaerobic conditions (Kim et al. *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim *J. Bacteriol.* 190:3851-3858 (2008); Zhou et al. *Biotechnol. Lett.* 30:335-342 (2008)). In contrast to the *E. coli* PDH, the *B. subtilis* complex is active and required for growth under anaerobic conditions (Nakano *J. Bacteriol.* 179:6749-6755 (1997)). The *Klebsiella pneumoniae* PDH, characterized during growth on glycerol, is also active under anaerobic conditions (Menzel et al. *J. Biotechnol.* 56:135-142 (1997)). Crystal structures of the enzyme complex from bovine kidney (Zhou et al. *Proc. Natl. Acad. Sci. U.S.A.* 98:14802-14807 (2001)) and the E2 catalytic domain from *Azotobacter vinelandii* are available (Mattevi et al. *Science* 255:1544-1550 (1992)). Some mammalian PDH enzymes complexes can react on alternate substrates such as 2-oxobutanoate, although comparative kinetics of *Rattus norvegicus* PDH and BCKAD indicate that BCKAD has higher activity on 2-oxobutanoate as a substrate (Paxton et al. *Biochem. J.* 234:295-303 (1986)).

aceE	NP_414656.1	*Escherichia coli* str. K12 substr. MG1655
aceF	NP_414657.1	*Escherichia coli* str. K12 substr. MG1655
lpd	NP_414658.1	*Escherichia coli* str. K12 substr. MG1655
pdhA	P21881.1	*Bacillus subtilis*
pdhB	P21882.1	*Bacillus subtilis*
pdhC	P21883.2	*Bacillus subtilis*
pdhD	P21880.1	*Bacillus subtilis*
aceE	YP_001333808.1	*Klebsiella pneumonia* MGH78578
aceF	YP_001333809.1	*Klebsiella pneumonia* MGH78578
lpdA	YP_001333810.1	*Klebsiella pneumonia* MGH78578
Pdha1	NP_001004072.2	*Rattus norvegicus*
Pdha2	NP_446446.1	*Rattus norvegicus*
Dlat	NP_112287.1	*Rattus norvegicus*
Dld	NP_955417.1	*Rattus norvegicus*

As an alternative to the large multienzyme 2-keto-acid dehydrogenase complexes described above, some anaerobic organisms utilize enzymes in the 2-ketoacid oxidoreductase family (OFOR) to catalyze acylating oxidative decarboxylation of 2-keto-acids. Unlike the dehydrogenase complexes, these enzymes contain iron-sulfur clusters, utilize different cofactors, and use ferredoxin or flavodixin as electron acceptors in lieu of NAD(P)H. While most enzymes in this family are specific to pyruvate as a substrate (POR) some 2-keto-acid:ferredoxin oxidoreductases have been shown to accept a broad range of 2-ketoacids as substrates including alpha-ketoglutarate and 2-oxobutanoate (Fukuda and Wakagi *Biochim. Biophys. Acta* 1597:74-80 (2002); Zhang et al. *J. Biochem.* 120:587-599 (1996)). One such enzyme is the OFOR from the thermoacidophilic archaeon *Sulfolobus tokodaii* 7, which contains an alpha and beta subunit encoded by gene ST2300 (Fukuda and Wakagi *Biochim. Biophys. Acta* 1597:74-80 (2002); Zhang et al. *J. Biochem.* 120:587-599 (1996)). A plasmid-based expression system has been developed for efficiently expressing this protein in *E. coli* (Fukuda et al. *Eur. J. Biochem.* 268:5639-5646 (2001)) and residues involved in substrate specificity were determined (Fukuda and Wakagi *Biochim. Biophys. Acta* 1597:74-80 (2002)). Two OFORs from *Aeropyrum pernix* str. K1 have also been recently cloned into *E. coli*, characterized, and found to react with a broad range of 2-oxoacids (Nishizawa et al. *FEBS Lett.* 579:2319-2322 (2005)). The gene sequences of these OFOR candidates are available, although they do not have GenBank identifiers assigned to date. There is bioinformatic evidence that similar enzymes are present in all archaea, some anaerobic bacteria and amitochondrial eukarya (Fukuda and Wakagi *Biochim. Biophys. Acta* 1597:74-80 (2005)). This class of enzyme is also interesting from an energetic standpoint, as reduced ferredoxin could be used to generate NADH by ferredoxin-NAD reductase (Petitdemange et al. *Biochim. Biophys. Acta* 421: 334-337 (1976)). Also, since most of the enzymes are designed to operate under anaerobic conditions, less enzyme engineering may be required relative to enzymes in the 2-keto-acid dehydrogenase complex family for activity in an anaerobic environment.

ST2300	NP_378302.1	*Sulfolobus tokodaii* 7

1.2.1.d—Oxidoreductase (Phosphorylating/Dephosphorylating)

Exemplary enzymes in this class include glyceraldehyde 3-phosphate dehydrogenase which converts glyceraldehyde-3-phosphate into D-glycerate 1,3-bisphosphate (for example, *E. coli* gapA (Branlant and Branlant *Eur. J. Biochem.* 150:61-66 (1985)), aspartate-semialdehyde dehydrogenase which converts L-aspartate-4-semialdehyde into L-4-aspartyl-phosphate (for example, *E. coli* asd (Biellmann et al. *Eur. J. Biochem.* 104:53-58 (1980)), N-acetyl-gamma-glutamyl-phosphate reductase which converts N-acetyl-L-glutamate-5-semialdehyde into N-acetyl-L-glutamyl-5-phosphate (for example, *E. coli* argC (Parsot et al. Gene 68:275-283 (1988)), and glutamate-5-semialdehyde dehydrogenase which converts L-glutamate-5-semialdehyde into L-glutamyl-5-phospate (for example, *E. coli* proA (Smith et al. *J. Bacteriol.* 157:545-551 (1984)).

gapA	P0A9B2.2	*Escherichia coli*
asd	NP_417891.1	*Escherichia coli*
argC	NP_418393.1	*Escherichia coli*
proA	NP_414778.1	*Escherichia coli*

1.3.1.a—Oxidoreductase Operating on CH—CH Donors

An exemplary enoyl-CoA reductase is the gene product of bcd from *C. acetobutylicum* (Atsumi et al. *Metab Eng* (2007); Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996), which naturally catalyzes the reduction of crotonyl-CoA to butyryl-CoA. Activity of this enzyme can

be enhanced by expressing bcd in conjunction with expression of the *C. acetobutylicum* etfAB genes, which encode an electron transfer flavoprotein. An additional candidate for the enoyl-CoA reductase step is the mitochondrial enoyl-CoA reductase from *E. gracilis* (Hoffmeister et al. *Journal of Biological Chemistry* 280:4329-4338 (2005)). A construct derived from this sequence following the removal of its mitochondrial targeting leader sequence was cloned in *E. coli* resulting in an active enzyme (Hoffmeister et al., supra, (2005)). This approach is well known to those skilled in the art of expressing eukarytotic genes, particularly those with leader sequences that may target the gene product to a specific intracellular compartment, in prokaryotic organisms. A close homolog of this gene, TDE0597, from the prokaryote *Treponema denticola* represents a third enoyl-CoA reductase which has been cloned and expressed in *E. coli* (Tucci and Martin *FEBS Letters* 581:1561-1566 (2007)).

bcd	NP_349317.1	*Clostridium acetobutylicum*
etfA	NP_349315.1	*Clostridium acetobutylicum*
etfB	NP_349316.1	*Clostridium acetobutylicum*
TER	Q5EU90.1	*Euglena gracilis*
TDE0597	NP_971211.1	*Treponema denticola*

Exemplary 2-enoate reductase (EC 1.3.1.31) enzymes are known to catalyze the NADH-dependent reduction of a wide variety of α,β-unsaturated carboxylic acids and aldehydes (Rohdich et al. *J. Biol. Chem.* 276:5779-5787 (2001)). 2-Enoate reductase is encoded by enr in several species of *Clostridia* (Giesel and Simon *Arch Microbiol.* 135(1): p. 51-57 (2001) including *C. tyrobutyricum*, and *C. thermoaceticum* (now called *Moorella thermoaceticum*) (Rohdich et al., supra, (2001)). In the recently published genome sequence of *C. kluyveri,* 9 coding sequences for enoate reductases have been reported, out of which one has been characterized (Seedorf et al. *Proc Natl Acad Sci U.S.A.* 105(6):2128-33 (2008)). The enr genes from both *C. tyrobutyricum* and *C. thermoaceticum* have been cloned and sequenced and show 59% identity to each other. The former gene is also found to have approximately 75% similarity to the characterized gene in *C. kluyveri* (Giesel and Simon *Arch Microbiol* 135(1):51-57 (1983)). It has been reported based on these sequence results that enr is very similar to the dienoyl CoA reductase in *E. coli* (fadH) (163 Rohdich et al., supra (2001)). The *C. thermoaceticum* enr gene has also been expressed in an enzymatically active form in *E. coli* (163 Rohdich et al., supra (2001)).

fadH	NP_417552.1	*Escherichia coli*
enr	ACA54153.1	*Clostridium botulinum* A3 str
enr	CAA71086.1	*Clostridium tyrobutyricum*
enr	CAA76083.1	*Clostridium kluyveri*
enr	YP_430895.1	*Moorella thermoacetica*

1.4.1.a—Oxidoreductase Operating on Amino Acids

Most oxidoreductases operating on amino acids catalyze the oxidative deamination of alpha-amino acids with NAD+ or NADP+ as acceptor. Exemplary oxidoreductases operating on amino acids include glutamate dehydrogenase (deaminating), encoded by gdhA, leucine dehydrogenase (deaminating), encoded by ldh, and aspartate dehydrogenase (deaminating), encoded by nadX. The gdhA gene product from *Escherichia coli* (Korber et al. *J. Mol. Biol.* 234:1270-1273 (1993); McPherson and Wootton *Nucleic. Acids Res.* 11:5257-5266 (1983)), gdh from *Thermotoga maritima* (Kort et al. *Extremophiles* 1:52-60 (1997); Lebbink, et al. *J. Mol. Biol.* 280:287-296 (1998)); Lebbink et al. *J. Mol. Biol.* 289:357-369 (1999)), and gdhA1 from *Halobacterium salinarum* (Ingoldsby et al. *Gene* 349:237-244 (2005)) catalyze the reversible interconversion of glutamate to 2-oxoglutarate and ammonia, while favoring NADP(H), NAD(H), or both, respectively. The ldh gene of *Bacillus cereus* encodes the LeuDH protein that has a wide of range of substrates including leucine, isoleucine, valine, and 2-aminobutanoate (Ansorge and Kula *Biotechnol Bioeng.* 68:557-562 (2000); Stoyan et al. *J. Biotechnol* 54:77-80 (1997)). The nadX gene from *Thermotoga maritime* encoding for the aspartate dehydrogenase is involved in the biosynthesis of NAD (Yang et al. *J. Biol. Chem.* 278:8804-8808 (2003)).

gdhA	P00370	*Escherichia coli*
gdh	P96110.4	*Thermotoga maritima*
gdhA1	NP_279651.1	*Halobacterium salinarum*
ldh	P0A393	*Bacillus cereus*
nadX	NP_229443.1	*Thermotoga maritima*

The lysine 6-dehydrogenase (deaminating), encoded by lysDH gene, catalyze the oxidative deamination of the ϵ-amino group of L-lysine to form 2-aminoadipate-6-semialdehyde, which in turn nonenzymatically cyclizes to form Δ1-piperideine-6-carboxylate (Misono and Nagasaki *J. Bacteriol.* 150:398-401 (1982)). The lysDH gene from *Geobacillus stearothermophilus* encodes a thermophilic NAD-dependent lysine 6-dehydrogenase (Heydari et al. *Appl Environ. Microbiol* 70:937-942 (2004)). In addition, the lysDH gene from *Aeropyrum pernix* K1 is identified through homology from genome projects.

lysDH	AB052732	*Geobacillus stearothermophilus*
lysDH	NP_147035.1	*Aeropyrum pernix* K1
ldh	P0A393	*Bacillus cereus*

2.3.1.a—Acyltransferase (Transferring Phosphate Group)

Exemplary phosphate transferring acyltransferases include phosphotransacetylase, encoded by pta, and phosphotransbutyrylase, encoded by ptb. The pta gene from *E. coli* encodes an enzyme that can convert acetyl-CoA into acetyl-phosphate, and vice versa (Suzuki, T. *Biochim. Biophys. Acta* 191:559-569 (1969)). This enzyme can also utilize propionyl-CoA instead of acetyl-CoA forming propionate in the process (Hesslinger et al. *Mol. Microbiol* 27:477-492 (1998)). Similarly, the ptb gene from *C. acetobutylicum* encodes an enzyme that can convert butyryl-CoA into butyryl-phosphate (Walter et al. *Gene* 134(1): p. 107-11 (1993)); Huang et al. *J Mol Microbiol Biotechnol* 2(1): p. 33-38 (2000). Additional ptb genes can be found in butyrate-producing bacterium L2-50 (Louis et al. *J. Bacteriol.* 186: 2099-2106 (2004)) and *Bacillus megaterium* (Vazquez et al. *Curr. Microbiol* 42:345-349 (2001)).

pta	NP_416800.1	*Escherichia coli*
ptb	NP_349676	*Clostridium acetobutylicum*
ptb	AAR19757.1	butyrate-producing bacterium L2-50
ptb	CAC07932.1	*Bacillus megaterium*

2.6.1.a—Aminotransferase

Aspartate aminotransferase transfers an amino group from aspartate to alpha-ketoglutarate, forming glutamate and oxaloacetate. This conversion is catalyzed by, for example, the gene products of aspC from *Escherichia coli*

(Yagi et al. *FEBS Lett.* 100:81-84 (1979); Yagi et al. *Methods Enzymol.* 113:83-89 (1985)), AAT2 from *Saccharomyces cerevisiae* (Yagi et al. *J Biochem.* 92:35-43 (1982)) and ASPS from *Arabidopsis thaliana* (48, 108, 225 48. de la et al. *Plant J* 46:414-425 (2006); Kwok and Hanson *J Exp. Bot.* 55:595-604 (2004); Wilkie and Warren *Protein Expr. Purif.* 12:381-389 (1998)). Valine aminotransferase catalyzes the conversion of valine and pyruvate to 2-ketoisovalerate and alanine. The *E. coli* gene, avtA, encodes one such enzyme (Whalen and Berg *J. Bacteriol.* 150:739-746 (1982)). This gene product also catalyzes the amination of α-ketobutyrate to generate α-aminobutyrate, although the amine donor in this reaction has not been identified (Whalen and Berg *J. Bacteriol.* 158:571-574 (1984)). The gene product of the *E. coli* serC catalyzes two reactions, phosphoserine aminotransferase and phosphohydroxythreonine aminotransferase (Lam and Winkler *J. Bacteriol.* 172:6518-6528 (1990)), and activity on non-phosphorylated substrates could not be detected (Drewke et al. *FEBS. Lett.* 390:179-182 (1996)).

aspC	NP_415448.1	*Escherichia coli*
AAT2	P23542.3	*Saccharomyces cerevisiae*
ASP5	P46248.2	*Arabidopsis thaliana*
avtA	YP_026231.1	*Escherichia coli*
serC	NP_415427.1	*Escherichia coli*

Cargill has developed a beta-alanine/alpha-ketoglutarate aminotransferase for producing 3-HP from beta-alanine via malonyl-semialdehyde (PCT/US2007/076252 (Jessen et al)). The gene product of SkPYD4 in *Saccharomyces kluyveri* was also shown to preferentially use beta-alanine as the amino group donor (Andersen et al. *FEBS. J.* 274:1804-1817 (2007)). SkUGA1 encodes a homologue of *Saccharomyces cerevisiae* GABA aminotransferase, UGA1 (Ramos et al. *Eur. J. Biochem.* 149:401-404 (1985)), whereas SkPYD4 encodes an enzyme involved in both β-alanine and GABA transamination (Andersen et al. *FEBS. J.* 274:1804-1817 (2007)). 3-Amino-2-methylpropionate transaminase catalyzes the transformation from methylmalonate semialdehyde to 3-amino-2-methylpropionate. The enzyme has been characterized in *Rattus norvegicus* and *Sus scrofa* and is encoded by Abat (Kakimoto et al. *Biochim. Biophys. Acta* 156:374-380 (1968); Tamaki et al. *Methods Enzymol.* 324:376-389 (2000)). Enzyme candidates in other organisms with high sequence homology to 3-amino-2-methylpropionate transaminase include Gta-1 in *C. elegans* and gabT in *Bacillus subtilus*. Additionally, one of the native GABA aminotransferases in *E. coli*, encoded by gene gabT, has been shown to have broad substrate specificity (Liu et al. *Biochemistry* 43:10896-10905 (2004); Schulz et al. *Appl Environ Microbiol* 56:1-6 (1990)). The gene product of puuE catalyzes the other 4-aminobutyrate transaminase in *E. coli* (Kurihara et al. *J. Biol. Chem.* 280:4602-4608 (2005).).

SkyPYD4	ABF58893.1	*Saccharomyces kluyveri*
SkUGA1	ABF58894.1	*Saccharomyces kluyveri*
UGA1	NP_011533.1	*Saccharomyces cerevisiae*
Abat	P50554.3	*Rattus norvegicus*
Abat	P80147.2	*Sus scrofa*
Gta-1	Q21217.1	*Caenorhabditis elegans*
gabT	P94427.1	*Bacillus subtilus*
gabT	P22256.1	*Escherichia coli* K12
puuE	NP_415818.1	*Escherichia coli* K12

The X-ray crystal structures of *E. coli* 4-aminobutyrate transaminase unbound and bound to the inhibitor were reported (Liu et al. *Biochemistry* 43:10896-10905 (2004)). The substrates binding and substrate specificities were studied and suggested. The roles of active site residues were studied by site-directed mutagenesis and X-ray crystallography (Liu et al. *Biochemistry* 44:2982-2992 (2005)). Based on the structural information, attempt was made to engineer *E. coli* 4-aminobutyrate transaminase with novel enzymatic activity. These studies provide a base for evolving transaminase activity for BDO pathways.

2.7.2.a—Phosphotransferase, Carboxyl Group Acceptor

Exemplary kinases include the *E. coli* acetate kinase, encoded by ackA (Skarstedt and Silverstein *J. Biol. Chem.* 251:6775-6783 (1976)), the *C. acetobutylicum* butyrate kinases, encoded by buk1 and buk2 (Walter et al. *Gene* 134(1):107-111 (1993) (Huang et al. *J Mol Microbiol Biotechnol* 2(1):33-38 (2000)], and the *E. coli* gamma-glutamyl kinase, encoded by proB (Smith et al. *J. Bacteriol.* 157:545-551 (1984)). These enzymes phosphorylate acetate, butyrate, and glutamate, respectively. The ackA gene product from *E. coli* also phosphorylates propionate (Hesslinger et al. *Mol. Microbiol* 27:477-492 (1998)).

ackA	NP_416799.1	*Escherichia coli*
buk1	NP_349675	*Clostridium acetobutylicum*
buk2	Q97II1	*Clostridium acetobutylicum*
proB	NP_414777.1	*Escherichia coli*

2.8.3.a—Coenzyme-A Transferase

In the CoA-transferase family, *E. coli* enzyme acyl-CoA: acetate-CoA transferase, also known as acetate-CoA transferase (EC 2.8.3.8), has been shown to transfer the CoA moiety to acetate from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Matthies and Schink *Appl Environ Microbiol* 58:1435-1439 (1992)), valerate (Vanderwinkel et al. *Biochem. Biophys. Res Commun.* 33:902-908 (1968)) and butanoate (Vanderwinkel, supra (1968)). This enzyme is encoded by atoA (alpha subunit) and atoD (beta subunit) in *E. coli* sp. K12 (Korolev et al. *Acta Crystallogr. D Biol Crystallogr.* 58:2116-2121 (2002); Vanderwinkel, supra (1968)) and actA and cg0592 in *Corynebacterium glutamicum* ATCC 13032 (Duncan et al. *Appl Environ Microbiol* 68:5186-5190 (2002)). Additional genes found by sequence homology include atoD and atoA in *Escherichia coli* UT189.

atoA	P76459.1	*Escherichia coli* K12
atoD	P76458.1	*Escherichia coli* K12
actA	YP_226809.1	*Corynebacterium glutamicum* ATCC 13032
cg0592	YP_224801.1	*Corynebacterium glutamicum* ATCC 13032
atoA	ABE07971.1	*Escherichia coli* UT189
atoD	ABE07970.1	*Escherichia coli* UT189

Similar transformations are catalyzed by the gene products of cat1, cat2, and cat3 of *Clostridium* kluyveri which have been shown to exhibit succinyl-CoA, 4-hydroxybutyryl-CoA, and butyryl-CoA acetyltransferase activity, respectively (Seedorf et al. *Proc Natl Acad Sci U.S.A.* 105(6):2128-2133 (2008); Sohling and Gottschalk *J Bacteriol* 178(3):871-880 (1996)].

cat1	P38946.1	*Clostridium kluyveri*
cat2	P38942.2	*Clostridium kluyveri*
cat3	EDK35586.1	*Clostridium kluyveri*

The glutaconate-CoA-transferase (EC 2.8.3.12) enzyme from anaerobic bacterium *Acidaminococcus fermentans* reacts with diacid glutaconyl-CoA and 3-butenoyl-CoA (Mack and Buckel *FEES Lett.* 405:209-212 (1997)). The genes encoding this enzyme are gctA and gctB. This enzyme has reduced but detectable activity with other CoA derivatives including glutaryl-CoA, 2-hydroxyglutaryl-CoA, adipyl-CoA and acrylyl-CoA (Buckel et al. *Eur. J. Biochem.* 118:315-321 (1981)). The enzyme has been cloned and expressed in *E. coli* (Mac et al. *Eur. J. Biochem.* 226:41-51 (1994)).

gctA	CAA57199.1	*Acidaminococcus fermentans*
gctB	CAA57200.1	*Acidaminococcus fermentans*

3.1.2.a—Thiolester Hydrolase (CoA Specific)

In the CoA hydrolase family, the enzyme 3-hydroxyisobutyryl-CoA hydrolase is specific for 3-HIBCoA and has been described to efficiently catalyze the desired transformation during valine degradation (Shimomura et al. *J Biol Chem* 269:14248-14253 (1994)). Genes encoding this enzyme include hibch of *Rattus norvegicus* (Shimomura et al., supra (1994); Shimomura et al. *Methods Enzymol.* 324:229-240 (2000) and *Homo sapiens* (Shimomura et al., supra, 2000). Candidate genes by sequence homology include hibch of *Saccharomyces cerevisiae* and BC 2292 of *Bacillus cereus*.

hibch	Q5XIE6.2	*Rattus norvegicus*
hibch	Q6NVY1.2	*Homo sapiens*
hibch	P28817.2	*Saccharomyces cerevisiae*
BC_2292	Q81DR3	*Bacillus cereus*

The conversion of adipyl-CoA to adipate can be carried out by an acyl-CoA hydrolase or equivalently a thioesterase. The top *E. coli* gene candidate is tesB (Naggert et al. *J Biol. Chem.* 266(17):11044-11050 (1991)] which shows high similarity to the human acot8 which is a dicarboxylic acid acetyltransferase with activity on adipyl-CoA (Westin et al. *J Biol Chem* 280(46): 38125-38132 (2005). This activity has also been characterized in the rat liver (Deana, *Biochem Int.* 26(4): p. 767-773 (1992)).

tesB	NP_414986	*Escherichia coli*
acot8	CAA15502	*Homo sapiens*
acot8	NP_570112	*Rattus norvegicus*

Other potential *E. coli* thiolester hydrolases include the gene products of tesA (Bonner and Bloch, *J Biol. Chem.* 247(10):3123-3133 (1972)), ybgC (Kuznetsova et al., *FEMS Microbiol Rev.* 29(2):263-279 (2005); Zhuang et al., *FEBS Lett.* 516(1-3):161-163 (2002)) paaI (Song et al., *J Biol. Chem.* 281(16):11028-11038 (2006)), and ybdB (Leduc et al., *J Bacteriol.* 189(19):7112-7126 (2007)).

tesA	NP_415027	*Escherichia coli*
ybgC	NP_415264	*Escherichia coli*
paaI	NP_415914	*Escherichia coli*
ybdB	NP_415129	*Escherichia coli*

Several eukaryotic acetyl-CoA hydrolases (EC 3.1.2.1) have broad substrate specificity. The enzyme from *Rattus norvegicus* brain (Robinson et al. *Biochem. Biophys. Res. Commun.* 71:959-965 (1976)) can react with butyryl-CoA, hexanoyl-CoA and malonyl-CoA.

acot12	NP_570103.1	*Rattus norvegicus*

4.1.1.a—Carboxy-Lyase

An exemplary carboxy-lyase is acetolactate decarboxylase which participates in citrate catabolism and branched-chain amino acid biosynthesis, converting 2-acetolactate to acetoin. In *Lactococcus lactis* the enzyme is composed of six subunits, encoded by gene aldB, and is activated by valine, leucine and isoleucine (Goupil et al. *Appl. Environ. Microbiol.* 62:2636-2640 (1996); Goupil-Feuillerat et al. *J. Bacteriol.* 182:5399-5408 (2000)). This enzyme has been overexpressed and characterized in *E. coli* (Phalip et al. *FEBS Lett.* 351:95-99 (1994)). In other organisms the enzyme is a dimer, encoded by aldC in *Streptococcus* thermophilus (Monnet et al. *Lett. Appl. Microbiol.* 36:399-405 (2003)), aldB in *Bacillus brevis* (Diderichsen et al. *J. Bacteriol.* 172:4315-4321 (1990); Najmudin et al. *Acta Crystallogr. D. Biol. Crystallogr.* 59:1073-1075 (2003)) and budA from *Enterobacter aerogenes* (Diderichsen et al. *J. Bacteriol.* 172:4315-4321 (1990)). The enzyme from *Bacillus brevis* was cloned and overexpressed in *Bacillus subtilis* and characterized crystallographically (Najmudin et al. *Acta Crystallogr. D. Biol. Crystallogr.* 59:1073-1075 (2003)). Additionally, the enzyme from *Leuconostoc lactis* has been purified and characterized but the gene has not been isolated (O'Sullivan et al. *FEMS Microbiol. Lett.* 194:245-249 (2001)).

aldB	NP_267384.1	*Lactococcus lactis*
aldC	Q8L208	*Streptococcus thermophilus*
aldB	P23616.1	*Bacillus brevis*
budA	P05361.1	*Enterobacter aerogenes*

Aconitate decarboxylase catalyzes the final step in itaconate biosynthesis in a strain of *Candida* and also in the filamentous fungus *Aspergillus terreus* (Bonnarme et al. *J Bacteriol.* 177:3573-3578 (1995); Willke and Vorlop *Appl Microbiol Biotechnol* 56:289-295 (2001)). Although itaconate is a compound of biotechnological interest, the aconitate decarboxylase gene or protein sequence has not been reported to date.

4-oxalocronate decarboxylase has been isolated from numerous organisms and characterized. Genes encoding this enzyme include dmpH and dmpE in *Pseudomonas* sp. (strain 600) (Shingler et al. *J. Bacteriol.* 174:711-724 (1992)), xylII and xylIII from *Pseudomonas putida* (Kato and Asano *Arch. Microbiol* 168:457-463 (1997); Lian and Whitman *J. Am. Chem. Soc.* 116:10403-10411 (1994); Stanley et al. *Biochemistry* 39:3514 (2000)) and Reut_B5691 and Reut_B5692 from *Ralstonia eutropha* JMP134 (Hughes et al. *J. Bacteriol.* 158:79-83 (1984)). The genes encoding the enzyme from *Pseudomonas* sp. (strain 600) have been cloned and expressed in *E. coli* (Shingler et al. *J. Bacteriol.* 174:711-724 (1992)).

dmpH	CAA43228.1	*Pseudomonas* sp. CF600
dmpE	CAA43225.1	*Pseudomonas* sp. CF600
xylII	YP_709328.1	*Pseudomonas putida*
xylIII	YP_709353.1	*Pseudomonas putida*
Reut_B5691	YP_299880.1	*Ralstonia eutropha* JMP134
Reut_B5692	YP_299881.1	*Ralstonia eutropha* JMP134

An additional class of decarboxylases has been characterized that catalyze the conversion of cinnamate (pheny-

lacrylate) and substituted cinnamate derivatives to the corresponding styrene derivatives. These enzymes are common in a variety of organisms and specific genes encoding these enzymes that have been cloned and expressed in *E. coli* are: pad 1 from *Saccharomyces cerevisae* (Clausen et al. *Gene* 142:107-112 (1994)), pdc from *Lactobacillus plantarum* (Barthelmebs et al. *Appl Environ Microbiol* 67:1063-1069 (2001); Qi et al. *Metab Eng* 9:268-276 (2007); Rodriguez et al. *J. Agric. Food Chem.* 56:3068-3072 (2008)), pofK (pad) from *Klebsiella oxytoca* (Hashidoko et al. *Biosci. Biotech. Biochem.* 58:217-218 (1994); Uchiyama et al. *Biosci. Biotechnol. Biochem.* 72:116-123 (2008)), *Pedicoccus pentosaceus* (Barthelmebs et al. *Appl Environ Microbiol* 67:1063-1069 (2001)), and padC from *Bacillus subtilis* and *Bacillus pumilus* (Lingen et al. *Protein Eng* 15:585-593 (2002)). A ferulic acid decarboxylase from *Pseudomonas fluorescens* also has been purified and characterized (Huang et al. *J. Bacteriol.* 176:5912-5918 (1994)). Importantly, this class of enzymes have been shown to be stable and do not require either exogenous or internally bound co-factors, thus making these enzymes ideally suitable for biotransformations (Sariaslani, *Annu. Rev. Microbiol.* 61:51-69 (2007)).

pad1	AB368798	*Saccharomyces cerevisae*
pdc	U63827	*Lactobacillus plantarum*
pofK (pad)	AB330293	*Klebsiella oxytoca*
padC	AF017117	*Bacillus subtilis*
pad	AJ276891	*Pedicoccus pentosaceus*
pad	AJ278683	*Bacillus pumilus*

Additional decarboxylase enzymes can form succinic semialdehyde from alpha-ketoglutarate. These include the alpha-ketoglutarate decarboxylase enzymes from *Euglena gracilis* (Shigeoka et al. *Biochem. J.* 282(Pt 2):319-323 (1992); Shigeoka and Nakano *Arch. Biochem. Biophys.* 288:22-28 (1991); Shigeoka and Nakano *Biochem. J.* 292 (Pt 2):463-467 (1993)), whose corresponding gene sequence has yet to be determined, and from *Mycobacterium tuberculosis* (Tian et al. *Proc Natl Acad Sci U.S.A.* 102:10670-10675 (2005)). In addition, glutamate decarboxylase enzymes can convert glutamate into 4-aminobutyrate such as the products of the *E. coli* gadA and gadB genes (De Biase et al. *Protein. Expr. Purif.* 8:430-438 (1993)).

kgd	O50463.4	*Mycobacterium tuberculosis*
gadA	NP_417974	*Escherichia coli*
gadB	NP_416010	*Escherichia coli*

Keto-Acid Decarboxylases

Pyruvate decarboxylase (PDC, EC 4.1.1.1), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. This enzyme has a broad substrate range for aliphatic 2-keto acids including 2-ketobutyrate, 2-ketovalerate, 3-hydroxypyruvate and 2-phenylpyruvate (Berg et al. *Science* 318:1782-1786 (2007)). The PDC from *Zymomonas mobilus*, encoded by pdc, has been a subject of directed engineering studies that altered the affinity for different substrates (Siegert et al. *Protein Eng Des Sel* 18:345-357 (2005)). The PDC from *Saccharomyces cerevisiae* has also been extensively studied, engineered for altered activity, and functionally expressed in *E. coli* (Killenberg-Jabs et al. *Eur. J. Biochem.* 268:1698-1704 (2001); L1 and Jordan *Biochemistry* 38:10004-10012 (1999); ter Schure et al. *Appl. Environ. Microbiol.* 64:1303-1307 (1998)). The crystal structure of this enzyme is available (Killenberg-Jabs *Eur. J. Biochem.* 268:1698-1704 (2001)). Other well-characterized PDC candidates include the enzymes from *Acetobacter* pasteurians (Chandra et al. *Arch. Microbiol.* 176:443-451 (2001)) and *Kluyveromyces lactis* (Krieger et al. *Eur. J. Biochem.* 269: 3256-3263 (2002)).

Gene	GenBank ID	Organism
pdc	P06672.1	*Zymomonas mobilus*
pdc1	P06169	*Saccharomyces cerevisiae*
pdc	Q8L388	*Acetobacter pasteurians*
pdc1	Q12629	*Kluyveromyces lactis*

Like PDC, benzoylformate decarboxylase (EC 4.1.1.7) has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Hasson et al. *Biochemistry* 37:9918-9930 (1998); Polovnikova et al. *Biochemistry* 42:1820-1830 (2003)). Site-directed mutagenesis of two residues in the active site of the *Pseudomonas putida* enzyme altered the affinity (Km) of naturally and non-naturally occurring substrates (Siegert *Protein Eng Des Sel* 18:345-357 (2005)). The properties of this enzyme have been further modified by directed engineering (Lingen et al. *Protein Eng* 15:585-593 (2002)); Lingen *Chembiochem* 4:721-726 (2003)). The enzyme from *Pseudomonas aeruginosa*, encoded by mdlC, has also been characterized experimentally (Barrowman et al. *FEMS Microbiology Letters* 34:57-60 (1986)). Additional gene candidates from *Pseudomonas stutzeri, Pseudomonas fluorescens* and other organisms can be inferred by sequence homology or identified using a growth selection system developed in *Pseudomonas putida* (Henning et al. *Appl. Environ. Microbiol.* 72:7510-7517 (2006)).

mdlC	P20906.2	*Pseudomonas putida*
mdlC	Q9HUR2.1	*Pseudomonas aeruginosa*
dpgB	ABN80423.1	*Pseudomonas stutzeri*
ilvB-1	YP_260581.1	*Pseudomonas fluorescens*

4.2.1.a—Hydro-Lyase

The 2-(hydroxymethyl)glutarate dehydratase of *Eubacterium* barkeri is an exemplary hydro-lyase. This enzyme has been studied in the context of nicotinate catabolism and is encoded by hmd (Alhapel et al. *Proc Natl Acad Sci USA* 103:12341-12346 (2006)). Similar enzymes with high sequence homology are found in *Bacteroides capillosus, Anaerotruncus colihominis*, and *Natranaerobius thermophilius*.

hmd	ABC88407.1	*Eubacterium barkeri*
BACCAP_02294	ZP_02036683.1	*Bacteroides capillosus* ATCC 29799
ANACOL_02527	ZP_02443222.1	*Anaerotruncus colihominis* DSM 17241
NtherDRAFT_2368	ZP_02852366.1	*Natranaerobius thermophilus* JW/NM-WN-LF

A second exemplary hydro-lyase is fumarate hydratase, an enzyme catalyzing the dehydration of malate to fumarate. A wealth of structural information is available for this enzyme and researchers have successfully engineered the enzyme to alter activity, inhibition and localization (Weaver, T. *Acta Crystallogr. D Biol Crystallogr.* 61:1395-1401 (2005)). Additional fumarate hydratases include those

encoded by fumC from *Escherichia coli* (Estevez et al. *Protein Sci.* 11:1552-1557 (2002); Hong and Lee *Biotechnol. Bioprocess Eng.* 9:252-255 (2004); Rose and Weaver *Proc Natl Acad Sci U.S.A* 101:3393-3397 (2004)), *Campylobacter jejuni* (Smith et al. *Int. J. Biochem. Cell Biol* 31:961-975 (1999)) and *Thermus thermophilus* (Mizobata et al. *Arch. Biochem. Biophys.* 355:49-55 (1998)), and fumH from *Rattus norvegicus* (Kobayashi et al. *J. Biochem.* 89:1923-1931 (1981)). Similar enzymes with high sequence homology include fum1 from *Arabidopsis thaliana* and fumC from *Corynebacterium glutamicum*.

fumC	P05042.1	*Escherichia coli* K12
fumC	O69294.1	*Campylobacter jejuni*
fumC	P84127	*Thermus thermophilus*
fumH	P14408.1	*Rattus norvegicus*
fum1	P93033.2	*Arabidopsis thaliana*
fumC	Q8NRN8.1	*Corynebacterium glutamicum*

Citramalate hydrolyase, also called 2-methylmalate dehydratase, converts 2-methylmalate to mesaconate. 2-Methylmalate dehydratase activity was detected in *Clostridium tetanomorphum, Morganella morganii, Citrobacter amalonaticus* in the context of the glutamate degradation VI pathway (Kato and Asano *Arch. Microbiol* 168:457-463 (1997)); however the genes encoding this enzyme have not been sequenced to date.

The gene product of crt from *C. acetobutylicum* catalyzes the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA (Atsumi et al. *Metab Eng.;* 29 (2007)); Boynton et al. *Journal of Bacteriology* 178:3015-3024 (1996)). The enoyl-CoA hydratases, phaA and phaB, of *P. putida* are believed to carry out the hydroxylation of double bonds during phenylacetate catabolism; (Olivera et al. *Proc Natl Acad Sci USA* 95(11):6419-6424 (1998)). The paaA and paaB from *P. fluorescens* catalyze analogous transformations (14 Olivera et al., supra, 1998). Lastly, a number of *Escherichia coli* genes have been shown to demonstrate enoyl-CoA hydratase functionality including maoC (Park and Lee *J Bacteriol* 185(18):5391-5397 (2003)), paaF (Park and Lee *Biotechnol Bioeng.* 86(6):681-686 (2004a)); Park and Lee *Appl Biochem Biotechnol.* 113-116: 335-346 (2004b)); Ismail et al. *Eur J Biochem* 270(14): p. 3047-3054 (2003), and paaG (Park and Lee, supra, 2004; Park and Lee supra, 2004b; Ismail et al., supra, 2003).

maoC	NP_415905.1	*Escherichia coli*
paaF	NP_415911.1	*Escherichia coli*
paaG	NP_415912.1	*Escherichia coli*
crt	NP_349318.1	*Clostridium acetobutylicum*
paaA	NP_745427.1	*Pseudomonas putida*
paaB	NP_745426.1	*Pseudomonas putida*
phaA	ABF82233.1	*Pseudomonas fluorescens*
phaB	ABF82234.1	*Pseudomonas fluorescens*

The *E. coli* genes fadA and fadB encode a multienzyme complex that exhibits ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase activities (Yang et al. *Biochemistry* 30(27): p. 6788-6795 (1991); Yang et al. *J Biol Chem* 265(18): p. 10424-10429 (1990); Yang et al. *J Biol Chem* 266(24): p. 16255 (1991); Nakahigashi and Inokuchi *Nucleic Acids Res* 18(16): p. 4937 (1990)). The fadI and fadJ genes encode similar functions and are naturally expressed only anaerobically (Campbell et al. *Mol Microbiol* 47(3): p. 793-805 (2003). A method for producing poly[(R)-3-hydroxybutyrate] in *E. coli* that involves activating fadB (by knocking out a negative regulator, fadR) and co-expressing a non-native ketothiolase (phaA from *Ralstonia eutropha*) has been described previously (Sato et al. *J Biosci Bioeng* 103(1): 38-44 (2007)). This work clearly demonstrates that a β-oxidation enzyme, in particular the gene product of fadB which encodes both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities, can function as part of a pathway to produce longer chain molecules from acetyl-CoA precursors.

fadA	YP_026272.1	*Escherichia coli*
fadB	NP_418288.1	*Escherichia coli*
fadI	NP_416844.1	*Escherichia coli*
fadJ	NP_416843.1	*Escherichia coli*
fadR	NP_415705.1	*Escherichia coli*

4.3.1.a—Ammonia-Lyase

Aspartase (EC 4.3.1.1), catalyzing the deamination of aspartate to fumarate, is a widespread enzyme in microorganisms, and has been characterized extensively (Viola, R. E. *Adv. Enzymol. Relat Areas Mol. Biol.* 74:295-341 (2000)). The crystal structure of the *E. coli* aspartase, encoded by aspA, has been solved (Shi et al. *Biochemistry* 36:9136-9144 (1997)). The *E. coli* enzyme has also been shown to react with alternate substrates aspartatephenylmethylester, asparagine, benzyl-aspartate and malate (Ma et al. *Ann N.Y. Acad Sci* 672:60-65 (1992)). In a separate study, directed evolution was been employed on this enzyme to alter substrate specificity (Asano et al. *Biomol. Eng* 22:95-101 (2005)). Enzymes with aspartase functionality have also been characterized in *Haemophilus influenzae* (Sjostrom et al. *Biochim. Biophys. Acta* 1324:182-190 (1997)), *Pseudomonas fluorescens* (Takagi et al. *J. Biochem.* 96:545-552 (1984)), *Bacillus subtilus* (Sjostrom et al. *Biochim. Biophys. Acta* 1324:182-190 (1997)) and *Serratia marcescens* (Takagi and Kisumi *J Bacteriol.* 161:1-6 (1985)).

aspA	NP_418562	*Escherichia coli* K12 subsp. MG1655
aspA	P44324.1	*Haemophilus influenzae*
aspA	P07346.1	*Pseudomonas fluorescens*
ansB	P26899.1	*Bacillus subtilus*
aspA	P33109.1	*Serratia marcescens*

3-methylaspartase (EC 4.3.1.2), also known as beta-methylaspartase or 3-methylaspartate ammonia-lyase, catalyzes the deamination of threo-3-methylasparatate to mesaconate. The 3-methylaspartase from *Clostridium tetanomorphum* has been cloned, functionally expressed in *E. coli*, and crystallized (Asuncion et al. *Acta Crystallogr. D Biol Crystallogr.* 57:731-733 (2001); Asuncion et al. *J Biol. Chem.* 277:8306-8311 (2002); Botting et al. *Biochemistry* 27:2953-2955 (1988); Goda et al. *Biochemistry* 31:10747-10756 (1992). In *Citrobacter amalonaticus*, this enzyme is encoded by BAA28709 (Kato and Asano *Arch. Microbiol* 168:457-463 (1997)). 3-Methylaspartase has also been crystallized from *E. coli* YG1002 (Asano and Kato *FEMS Microbiol Lett.* 118:255-258 (1994)) although the protein sequence is not listed in public databases such as GenBank. Sequence homology can be used to identify additional candidate genes, including CTC_02563 in *C. tetani* and ECs0761 in *Escherichia coli* O157:H7.

MAL	AAB24070.1	*Clostridium tetanomorphum*
BAA28709	BAA28709.1	*Citrobacter amalonaticus*
CTC_02563	NP_783085.1	*Clostridium tetani*
ECs0761	BAB34184.1	*Escherichia coli* O157:H7 str. Sakai

Ammonia-lyase enzyme candidates that form enoyl-CoA products include beta-alanyl-CoA ammonia-lyase (EC 4.3.1.6), which deaminates beta-alanyl-CoA, and 3-aminobutyryl-CoA ammonia-lyase (EC 4.3.1.14). Two beta-alanyl-CoA ammonia lyases have been identified and characterized in *Clostridium propionicum* (Herrmann et al. *FEBS J.* 272:813-821 (2005)). No other beta-alanyl-CoA ammonia lyases have been studied to date, but gene candidates can be identified by sequence similarity. One such candidate is MXAN_4385 in *Myxococcus xanthus*.

acl2	CAG29275.1	*Clostridium propionicum*
acl1	CAG29274.1	*Clostridium propionicum*
MXAN_4385	YP_632558.1	*Myxococcus xanthus*

5.3.3.a—Isomerase

The 4-hydroxybutyryl-CoA dehydratases from both *Clostridium aminobutyrium* and *C. kluyveri* catalyze the reversible conversion of 4-hydroxybutyryl-CoA to crotonyl-CoA and posses an intrinsic vinylacetyl-CoA A-isomerase activity (Scherf and Buckel *Eur. J Biochem.* 215:421-429 (1993); Scherf et al. *Arch. Microbiol* 161:239-245 (1994)). Both native enzymes were purified and characterized, including the N-terminal amino acid sequences (Scherf and Buckel, supra, 1993; Scherf et al., supra, 1994). The abfD genes from *C. aminobutyrium* and *C. kluyveri* match exactly with these N-terminal amino acid sequences, thus are encoding the 4-hydroxybutyryl-CoA dehydratases/vinylacetyl-CoA A-isomerase. In addition, the abfD gene from *Porphyromonas gingivalis* ATCC 33277 is identified through homology from genome projects.

abfD	YP_001396399.1	*Clostridium kluyveri* DSM 555
abfD	P55792	*Clostridium aminobutyricum*
abfD	YP_001928843	*Porphyromonas gingivalis* ATCC 33277

5.4.3.a—Aminomutase

Lysine 2,3-aminomutase (EC 5.4.3.2) is an exemplary aminomutase that converts lysine to (3S)-3,6-diaminohexanoate, shifting an amine group from the 2- to the 3-position. The enzyme is found in bacteria that ferment lysine to acetate and butyrate, including as *Fusobacterium nuleatum* (kamA) (Barker et al. *J. Bacteriol.* 152:201-207 (1982)) and *Clostridium subterminale* (kamA) (Chirpich et al. *J. Biol. Chem.* 245:1778-1789 (1970)). The enzyme from *Clostridium subterminale* has been crystallized (Lepore et al. *Proc. Natl. Acad. Sci. U.S.A* 102:13819-13824 (2005)). An enzyme encoding this function is also encoded by yodO in *Bacillus subtilus* (Chen et al. *Biochem. J.* 348 Pt 3:539-549 (2000)). The enzyme utilizes pyridoxal 5'-phosphate as a cofactor, requires activation by S-Adenosylmethoionine, and is stereoselective, reacting with the only with L-lysine. The enzyme has not been shown to react with alternate substrates.

yodO	O34676.1	*Bacillus subtilus*
kamA	Q9XBQ8.1	*Clostridium subterminale*
kamA	Q8RHX4	*Fusobacterium nuleatum* subsp. *nuleatum*

A second aminomutase, beta-lysine 5,6-aminomutase (EC 5.4.3.3), catalyzes the next step of lysine fermentation to acetate and butyrate, which transforms (3S)-3,6-diaminohexanoate to (3S,5S)-3,5-diaminohexanoate, shifting a terminal amine group from the 6- to the 5-position. This enzyme also catalyzes the conversion of lysine to 2,5-diaminohexanoate and is also called lysine-5,6-aminomutase (EC 5.4.3.4). The enzyme has been crystallized in *Clostridium sticklandii* (kamD, kamE) (Berkovitch et al. *Proc. Natl. Acad. Sci. U.S.A* 101:15870-15875 (2004)). The enzyme from *Porphyromonas gingivalis* has also been characterized (Tang et al. *Biochemistry* 41:8767-8776 (2002)).

kamD	AAC79717.1	*Clostridium sticklandii*
kamE	AAC79718.1	*Clostridium sticklandii*
kamD	NC_002950.2	*Porphyromonas gingivalis* W83
kamE	NC_002950.2	*Porphyromonas gingivalis* W83

Ornithine 4,5-aminomutase (EC 5.4.3.5) converts D-ornithine to 2,4-diaminopentanoate, also shifting a terminal amine to the adjacent carbon. The enzyme from *Clostridium sticklandii* is encoded by two genes, oraE and oraS, and has been cloned, sequenced and expressed in *E. coli* (Chen et al. *J. Biol. Chem.* 276:44744-44750 (2001)). This enzyme has not been characterized in other organisms to date.

oraE	AAK72502	*Clostridium sticklandii*
oraS	AAK72501	*Clostridium sticklandii*

Tyrosine 2,3-aminomutase (EC 5.4.3.6) participates in tyrosine biosynthesis, reversibly converting tyrosine to 3-amino-3-(4-hdyroxyphenyl)propanoate by shifting an amine from the 2- to the 3-position. In *Streptomyces globisporus* the enzyme has also been shown to react with tyrosine derivatives (Christenson et al. *Biochemistry* 42:12708-12718 (2003)). Sequence information is not available.

Leucine 2,3-aminomutase (EC 5.4.3.7) converts L-leucine to beta-leucine during leucine degradation and biosynthesis. An assay for leucine 2,3-aminomutase detected activity in many organisms (Poston, J. M. *Methods Enzymol.* 166:130-135 (1988)) but genes encoding the enzyme have not been identified to date.

Cargill has developed a novel 2,3-aminomutase enzyme to convert L-alanine to β-alanine, thus creating a pathway from pyruvate to 3-HP in four biochemical steps (Liao et al., U.S. Publication No. 2005-0221466).

6.2.1.a—Acid-Thiol Ligase

An exemplary acid-thiol ligase is the gene products of sucCD of *E. coli* which together catalyze the formation of succinyl-CoA from succinate with the concaminant consumption of one ATP, a reaction which is reversible in vivo (Buck et al. *Biochemistry* 24(22): p. 6245-6252 (1985)). Additional exemplary CoA-ligases include the rat dicarboxylate-CoA ligase for which the sequence is yet uncharacterized (Vamecq et al. *Biochem J.* 230(3): p. 683-693 (1985)), either of the two characterized phenylacetate-CoA ligases from *P. chrysogenum* (Lamas-Maceiras et al. *Biochem J* 395(1):147-155 (2006); Wang et al. *Biochem Biophys Res Commun,* 360(2):453-458 (2007)), the phenylacetate-CoA ligase from *Pseudomonas putida* (Martinez-Blanco et al. *J Biol. Chem.* 265(12):7084-7090 (1990)), and the 6-carboxyhexanoate-CoA ligase from *Bacillus subtilis* (Bower et al. *J Bacteriol* 178(14):4122-4130 (1996)).

sucC	NP_415256.1	*Escherichia coli*
sucD	AAC73823.1	*Escherichia coli*
phl	CAJ15517.1	*Penicillium chrysogenum*
phlB	ABS19624.1	*Penicillium chrysogenum*
paaF	AAC24333.2	*Pseudomonas putida*
bioW	NP_390902.2	*Bacillus subtilis*

EXAMPLE V

Exemplary BDO Pathway from Succinyl-CoA

This example describes exemplary BDO pathways from succinyl-CoA.

BDO pathways from succinyl-CoA are described herein and have been described previously (see U.S. application Ser. No. 12/049,256, filed Mar. 14, 2008, and PCT application serial No. US08/57168, filed Mar. 14, 2008, each of which is incorporated herein by reference). Additional pathways are shown in FIG. **8**A. Enzymes of such exemplary BDO pathways are listed in Table 15, along with exemplary genes encoding these enzymes.

Briefly, succinyl-CoA can be converted to succinic semialdehyde by succinyl-CoA reductase (or succinate semialdehyde dehydrogenase) (EC 1.2.1.b). Succinate semialdehyde can be converted to 4-hydroxybutyrate by 4-hydroxybutyrate dehydrogenase (EC 1.1.1.a), as previously described. Alternatively, succinyl-CoA can be converted to 4-hydroxybutyrate by succinyl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), as previously described, or by 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a) or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). Alternatively, 4-hydroxybutyrate can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyrate kinase (EC 2.7.2.a), as previously described. 4-Hydroxybutyryl-phosphate can be converted to 4-hydroxybutyryl-CoA by phosphotrans-4-hydroxybutyrylase (EC 2.3.1.a), as previously described. Alternatively, 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutanal dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-Hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). Alternatively, 4-hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a), as previously described.

TABLE 15

BDO pathway from succinyl-CoA.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8A	1.2.1.b	succinyl-CoA	succinic semialdehyde	succinyl-CoA reductase (or succinate semialdehyde dehydrogenase)	sucD	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8A	1.1.1.a	succinate semialdehyde	4-hydroxybutyrate	4-hydroxybutyrate dehydrogenase	4hbd	YP_726053.1	*Ralstonia eutropha* H16	4-hydroxybutyrate
					4hbd	L21902.1	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate
					4hbd	Q94B07	*Arabidopsis thaliana*	4-hydroxybutyrate
8A	1.1.1.c	succinyl-CoA	4-hydroxybutyrate	succinyl-CoA reductase (alcohol forming)	adhE2	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8A	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA transferase	cat1, cat2, cat3	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
8A	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA hydrolase	tesB	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
8A	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	sucCD	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate

TABLE 15-continued

BDO pathway from succinyl-CoA.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8A	2.7.2.a	4-hydroxybutyrate	4-hydroxybutyryl-phosphate	4-hydroxybutyrate kinase	ackA	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					buk2	Q97II1	*Clostridium acetobutylicum*	butyrate
8A	2.3.1.a	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-CoA	phosphotrans-4-hydroxy-butyrylase	ptb	NP_349676	*Clostridium acetobutylicum*	butyryl-phosphate
					ptb	AAR19757.1	butyrate-producing *bacterium* L2-50	butyryl-phosphate
					ptb	CAC07932.1	*Bacillus megaterium*	butyryl-phosphate
8A	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutanal dehydrogenase (phosphorylating)	asd	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
8A	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	sucD	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8A	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	adhE2	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8A	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

EXAMPLE VI

Additional Exemplary BDO Pathways from Alpha-Ketoglutarate

This example describes exemplary BDO pathways from alpha-ketoglutarate.

BDO pathways from succinyl-CoA are described herein and have been described previously (see U.S. application Ser. No. 12/049,256, filed Mar. 14, 2008, and PCT application serial No. US08/57168, filed Mar. 14, 2008, each of which is incorporated herein by reference). Additional pathways are shown in FIG. **8**B. Enzymes of such exemplary BDO pathways are listed in Table 16, along with exemplary genes encoding these enzymes.

Briefly, alpha-ketoglutarate can be converted to succinic semialdehyde by alpha-ketoglutarate decarboxylase (EC 4.1.1.a), as previously described. Alternatively, alpha-ketoglutarate can be converted to glutamate by glutamate dehydrogenase (EC 1.4.1.a). 4-Aminobutyrate can be converted to succinic semialdehyde by 4-aminobutyrate oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutyrate transaminase (EC 2.6.1.a). Glutamate can be converted to 4-aminobutyrate by glutamate decarboxylase (EC 4.1.1.a). Succinate semialdehyde can be converted to 4-hydroxybutyrate by 4-hydroxybutyrate dehydrogenase (EC 1.1.1.a), as previously described. 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), as previously described, or by 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyrate kinase (EC 2.7.2.a). 4-Hydroxybutyryl-phosphate can be converted to 4-hydroxybutyryl-CoA by phosphotrans-4-hydroxybutyrylase (EC 2.3.1.a), as previously described. Alternatively, 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutanal dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-Hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b), as previously described. 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a), as previously described.

TABLE 16

BDO pathway from alpha-ketoglutarate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8B	4.1.1.a	alpha-ketoglutarate	succinic semialdehyde	alpha-ketoglutarate decarboxylase	kgd	O50463.4	*Mycobacterium tuberculosis*	alpha-ketoglutarate
					gadA	NP_417974	*Escherichia coli*	glutamate
					gadB	NP_416010	*Escherichia coli*	glutamate
8B	1.4.1.a	alpha-ketoglutarate	glutamate	glutamate dehydrogenase	gdhA	P00370	*Escherichia coli*	glutamate
					gdh	P96110.4	*Thermotoga maritima*	glutamate
					gdhA1	NP_279651.1	*Halobacterium salinarum*	glutamate
8B	1.4.1.a	4-aminobutyrate	succinic semialdehyde	4-aminobutyrate oxidoreductase (deaminating)	lysDH	AB052732	*Geobacillus stearothermophilus*	lysine
					lysDH	NP_147035.1	*Aeropyrum pernix* K1	lysine
					ldh	P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
8B	2.6.1.a	4-aminobutyrate	succinic semialdehyde	4-aminobutyrate transaminase	gabT	P22256.1	*Escherichia coli*	4-aminobutyryate
					puuE	NP_415818.1	*Escherichia coli*	4-aminobutyryate
					UGA1	NP_011533.1	*Saccharomyces cerevisiae*	4-aminobutyryate
8B	4.1.1.a	glutamate	4-aminobutyrate	glutamate decarboxylase	gadA	NP_417974	*Escherichia coli*	glutamate
					gadB	NP_416010	*Escherichia coli*	glutamate
					kgd	O50463.4	*Mycobacterium tuberculosis*	alpha-ketoglutarate
8B	1.1.1.a	succinate semialdehyde	4-hydroxybutyrate	4-hydroxybutyrate dehydrogenase	4hbd	YP_726053.1	*Ralstonia eutropha* H16	4-hydroxybutyrate
					4hbd	L21902.1	*Clostridium kluyveri* DSM 555	4-hydroxybutyrate
					4hbd	Q94B07	*Arabidopsis thaliana*	4-hydroxybutyrate
8B	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA transferase	cat1, cat2, cat3	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
					gctA, gctB	CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
					atoA, atoD	P76459.1, P76458.1	*Escherichia coli*	butanoate
8B	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA hydrolase	tesB	NP_414986	*Escherichia coli*	adipyl-CoA
					acot12	NP_570103.1	*Rattus norvegicus*	butyryl-CoA
					hibch	Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
8B	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	sucCD	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
					phl	CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
					bioW	NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
8B	2.7.2.a	4-hydroxybutyrate	4-hydroxybutyryl-phosphate	4-hydroxybutyrate kinase	ackA	NP_416799.1	*Escherichia coli*	acetate, propionate
					buk1	NP_349675	*Clostridium acetobutylicum*	butyrate
					buk2	Q97II1	*Clostridium acetobutylicum*	butyrate
8B	2.3.1.a	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-CoA	phosphotrans-4-hydroxy-butyrylase	ptb	NP_349676	*Clostridium acetobutylicum*	butyryl-phosphate
					ptb	AAR19757.1	butyrate-producing *bacterium* L2-50	butyryl-phosphate
					ptb	CAC07932.1	*Bacillus megaterium*	butyryl-phosphate

TABLE 16-continued

BDO pathway from alpha-ketoglutarate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name	GenBank ID (if available)	Organism	Known Substrates
8B	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutanal dehydrogenase (phosphorylating)	asd	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
					proA	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
					gapA	P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
8B	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	sucD	P38947.1	*Clostridium kluyveri*	succinyl-CoA
					sucD	NP_904963.1	*Porphyromonas gingivalis*	succinyl-CoA
					Msed_0709	YP_001190808.1	*Metallosphaera sedula*	malonyl-CoA
8B	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	adhE2	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
					mcr	AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
					FAR	AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8B	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2	NP_014032.1	*Saccharymyces cerevisiae*	general
					yqhD	NP_417484.1	*Escherichia coli*	>C3
					4hbd	L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

EXAMPLE VII

BDO Pathways from 4-Aminobutyrate

This example describes exemplary BDO pathway d from 4-aminobutyrate.

FIG. **9**A depicts exemplary BDO pathways in which 4-aminobutyrate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 17, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to 4-aminobutyryl-CoA by 4-aminobutyrate CoA transferase (EC 2.8.3.a), 4-aminobutyryl-CoA hydrolase (EC 3.1.2.a), or 4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase) (EC 6.2.1.a). 4-aminobutyryl-CoA can be converted to 4-oxobutyryl-CoA by 4-aminobutyryl-CoA oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutyryl-CoA transaminase (EC 2.6.1.a). 4-oxobutyryl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.a). 4-hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 17

BDO pathway from 4-aminobutyrate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
9A	2.8.3.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate CoA transferase	cat1, cat2, cat3
					gctA, gctB
					atoA, atoD
9A	3.1.2.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyryl-CoA hydrolase	tesB
					acot12
					hibch
9A	6.2.1.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase)	sucCD
					phl
					bioW
9A	1.4.1.a	4-aminobutyryl-CoA	4-oxobutyryl-CoA	4-aminobutyryl-CoA oxidoreductase (deaminating)	lysDH
					lysDH
					ldh

TABLE 17-continued

BDO pathway from 4-aminobutyrate.

9A	2.6.1.a	4-aminobutyryl-CoA	4-oxobutyryl-CoA	4-aminobutyryl-CoA transaminase	gabT abat SkyPYD4
9A	1.1.1.a	4-oxobutyryl-CoA	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA dehydrogenase	ADH2 yqhD 4hbd
8	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	adhE2 mcr FAR
8	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	sucD sucD Msed_0709
8	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2 yqhD 4hbd

FIG.	EC class	GenBank ID (if available)	Organism	Known Substrates
9A	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
9A	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
9A	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
9A	1.4.1.a	AB052732	*Geobacillus stearothermophilus*	lysine
		NP_147035.1	*Aeropyrum pernix* K1	lysine
		P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9A	2.6.1.a	P22256.1	*Escherichia coli*	4-aminobutyryate
		P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
		ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9A	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
8	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
8	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

Enzymes for another exemplary BDO pathway converting 4-aminobutyrate to BDO is shown in FIG. **9**A. Enzymes of such an exemplary BDO pathway are listed in Table 18, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to 4-aminobutyryl-CoA by 4-aminobutyrate CoA transferase (EC 2.8.3.a), 4-aminobutyryl-CoA hydrolase (EC 3.1.2.a) or 4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase) (EC 6.2.1.a). 4-aminobutyryl-CoA can be converted to 4-aminobutan-1-ol by 4-aminobutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-aminobutyryl-CoA can be converted to 4-aminobutanal by 4-aminobutyryl-CoA reductase (or 4-aminobutanal dehydrogenase) (EC 1.2.1.b), and 4-aminobutanal converted to 4-aminobutan-1-ol by 4-aminobutan-1-ol dehydrogenase (EC 1.1.1.a). 4-aminobutan-1-ol can be converted to 4-hydroxybutanal by 4-aminobutan-1-ol oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutan-1-ol transaminase (EC 2.6.1.a). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 18

BDO pathway from 4-aminobutyrate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
9A	2.8.3.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate CoA transferase	cat1, cat2, cat3
					gctA, gctB
					atoA, atoD
9A	3.1.2.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyryl-CoA hydrolase	tesB
					acot12
					hibch
9A	6.2.1.a	4-aminobutyrate	4-aminobutyryl-CoA	4-aminobutyrate-CoA ligase (or 4-aminobutyryl-CoA synthetase)	sucCD
					phl
					bioW
9A	1.1.1.c	4-aminobutyryl-CoA	4-aminobutan-1-ol	4-aminobutyryl-CoA reductase (alcohol forming)	adhE2
					mcr
					FAR
9A	1.2.1.b	4-aminobutyryl-CoA	4-aminobutanal	4-aminobutyryl-CoA reductase (or 4-aminobutanal dehydrogenase)	sucD
					sucD
					Msed_0709
9A	1.1.1.a	4-aminobutanal	4-aminobutan-1-ol	4-aminobutan-1-ol dehydrogenase	ADH2
					yqhD
					4hbd
9A	1.4.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol oxidoreductase (deaminating)	lysDH
					lysDH
					ldh
9A	2.6.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol transaminase	gabT
					abat
					SkyPYD4
9A	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2
					yqhD
					4hbd

FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
9A	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
9A	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA

TABLE 18-continued

BDO pathway from 4-aminobutyrate.

9A	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
9A	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
9A	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
9A	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
9A	1.4.1.a	AB052732	*Geobacillus stearothermophilus*	lysine
		NP_147035.1	*Aeropyrum pernix* K1	lysine
		P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9A	2.6.1.a	P22256.1	*Escherichia coli*	4-aminobutyryate
		P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
		ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9A	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

FIG. **9**B depicts exemplary BDO pathway in which 4-aminobutyrate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 19, along with exemplary genes encoding these enzymes.

Briefly, 4-aminobutyrate can be converted to [(4-aminobutanolyl)oxy]phosphonic acid by 4-aminobutyrate kinase (EC 2.7.2.a). [(4-aminobutanolyl)oxy]phosphonic acid can be converted to 4-aminobutanal by 4-aminobutyraldehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-aminobutanal can be converted to 4-aminobutan-1-ol by 4-aminobutan-1-ol dehydrogenase (EC 1.1.1.a). 4-aminobutan-1-ol can be converted to 4-hydroxybutanal by 4-aminobutan-1-ol oxidoreductase (deaminating) (EC 1.4.1.a) or 4-aminobutan-1-ol transaminase (EC 2.6.1.a). Alternatively, [(4-aminobutanolyl)oxy]phosphonic acid can be converted to [(4-oxobutanolyl)oxy]phosphonic acid by [(4-aminobutanolyl)oxy]phosphonic acid oxidoreductase (deaminating) (EC 1.4.1.a) or [(4-aminobutanolyl)oxy]phosphonic acid transaminase (EC 2.6.1.a). [(4-oxobutanolyl)oxy]phosphonic acid can be converted to 4-hydroxybutyryl-phosphate by 4-hydroxybutyryl-phosphate dehydrogenase (EC 1.1.1.a). 4-hydroxybutyryl-phosphate can be converted to 4-hydroxybutanal by 4-hydroxybutyraldehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 19

BDO pathway from 4-aminobutyrate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
9B	2.7.2.a	4-aminobutyrate	[(4-aminobutanolyl)oxy] phosphonic acid	4-aminobutyrate kinase	ackA
					buk1
					proB

TABLE 19-continued

BDO pathway from 4-aminobutyrate.

9B	1.2.1.d	[(4-aminobutanolyl) oxy] phosphonic acid	4-aminobutanal	4-aminobutyraldehyde dehydrogenase (phosphorylating)	asd
					proA
					gapA
9B	1.1.1.a	4-aminobutanal	4-aminobutan-1-ol	4-aminobutan-1-ol dehydrogenase	ADH2
					yqhD
					4hbd
9B	1.4.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol oxidoreductase (deaminating)	lysDH
					lysDH
					ldh
9B	2.6.1.a	4-aminobutan-1-ol	4-hydroxybutanal	4-aminobutan-1-ol transaminase	gabT
					abat
					SkyPYD4
9B	1.4.1.a	[(4-aminobutanolyl) oxy] phosphonic acid	[(4-oxobutanolyl) oxy] phosphonic acid	[(4-aminobutanolyl) oxy]phosphonic acid oxidoreductase (deaminating)	lysDH
					lysDH
					ldh
9B	2.6.1.a	[(4-aminobutanolyl) oxy] phosphonic acid	[(4-oxobutanolyl) oxy] phosphonic acid	[(4-aminobutanolyl) oxy]phosphonic acid transaminase	gabT
					SkyPYD4
					serC
9B	1.1.1.a	[(4-oxobutanolyl)oxy] phosphonic acid	4-hydroxybutyryl-phosphate	4-hydroxybutyryl-phosphate dehydrogenase	ADH2
					yqhD
					4hbd
9B	1.2.1.d	4-hydroxybutyryl-phosphate	4-hydroxybutanal	4-hydroxybutyraldehyde dehydrogenase (phosphorylating)	asd
					proA
					gapA
9B	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2
					yqhD
					4hbd

	FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
	9B	2.7.2.a	NP_416799.1	*Escherichia coli*	acetate, propionate
			NP_349675	*Clostridium acetobutylicum*	butyrate
			NP_414777.1	*Escherichia coli*	glutamate
	9B	1.2.1.d	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
			NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
			P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
	9B	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
			NP_417484.1	*Escherichia coli*	>C3
			L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
	9B	1.4.1.a	AB052732	*Geobacillus stearothermophilus*	lysine
			NP_147035.1	*Aeropyrum pernix* K1	lysine
			P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate

TABLE 19-continued

BDO pathway from 4-aminobutyrate.

9B	2.6.1.a	P22256.1	*Escherichia coli*	4-aminobutyryate
		P50554.3	*Rattus norvegicus*	3-amino-2-methylpropionate
		ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
9B	1.4.1.a	AB052732	*Geobacillus stearothermophilus*	lysine
		NP_147035.1	*Aeropyrum pernix* K1	lysine
		P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
9B	2.6.1.a	P22256.1	*Escherichia coli*	4-aminobutyryate
		ABF58893.1	*Saccharomyces kluyveri*	beta-alanine
		NP_415427.1	*Escherichia coli*	phosphoserine, phosphohydroxythreonine
9B	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
9B	1.2.1.d	NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
		NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
		P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
9B	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

FIG. **9**C shows an exemplary pathway through acetoacetate.

EXAMPLE VIII

Exemplary BDO Pathways from Alpha-Ketoglutarate

This example describes exemplary BDO pathways from alpha-ketoglutarate.

FIG. **10** depicts exemplary BDO pathways in which alpha-ketoglutarate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 20, along with exemplary genes encoding these enzymes.

Briefly, alpha-ketoglutarate can be converted to alpha-ketoglutaryl-phosphate by alpha-ketoglutarate 5-kinase (EC 2.7.2.a). Alpha-ketoglutaryl-phosphate can be converted to 2,5-dioxopentanoic acid by 2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). 2,5-dioxopentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 2,5-dioxopentanoic acid reductase (EC 1.1.1.a). Alternatively, alpha-ketoglutarate can be converted to alpha-ketoglutaryl-CoA by alpha-ketoglutarate CoA transferase (EC 2.8.3.a), alpha-ketoglutaryl-CoA hydrolase (EC 3.1.2.a) or alpha-ketoglutaryl-CoA ligase (or alpha-ketoglutaryl-CoA synthetase) (EC 6.2.1.a). Alpha-ketoglutaryl-CoA can be converted to 2,5-dioxopentanoic acid by alpha-ketoglutaryl-CoA reductase (or 2,5-dioxopentanoic acid dehydrogenase) (EC 1.2.1.b). 2,5-Dioxopentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 5-hydroxy-2-oxopentanoic acid dehydrogenase. Alternatively, alpha-ketoglutaryl-CoA can be converted to 5-hydroxy-2-oxopentanoic acid by alpha-ketoglutaryl-CoA reductase (alcohol forming) (EC 1.1.1.c). 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutanal by 5-hydroxy-2-oxopentanoic acid decarboxylase (EC 4.1.1.a). 4-hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a). 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutyryl-CoA by 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (EC 1.2.1.c).

TABLE 20

BDO pathway from alpha-ketoglutarate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
10	2.7.2.a	alpha-ketoglutarate	alpha-ketoglutaryl-phosphate	alpha-ketoglutarate 5-kinase	ackA
					buk1
					proB
10	1.2.1.d	alpha-ketoglutaryl-phosphate	2,5-dioxopentanoic acid	2,5-dioxopentanoic semialdehyde dehydrogenase (phosphorylating)	proA
					asd
					gapA

TABLE 20-continued

BDO pathway from alpha-ketoglutarate.

10	1.1.1.a	2,5-dioxopentanoic acid	5-hydroxy-2-oxopentanoic acid	2,5-dioxopentanoic acid reductase	ADH2 yqhD 4hbd
10	2.8.3.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutarate CoA transferase	cat1, cat2, cat3 gctA, gctB atoA, atoD
10	3.1.2.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutaryl-CoA hydrolase	tesB acot12 hibch
10	6.2.1.a	alpha-ketoglutarate	alpha-ketoglutaryl-CoA	alpha-ketoglutaryl-CoA ligase (or alpha-ketoglutaryl-CoA synthetase)	sucCD phl bioW
10	1.2.1.b	alpha-ketoglutaryl-CoA	2,5-dioxopentanoic acid	alpha-ketoglutaryl-CoA reductase (or 2,5-dioxopentanoic acid dehydrogenase)	sucD Msed_0709 bphG
10	1.1.1.a	2,5-dioxopentanoic acid	5-hydroxy-2-oxopentanoic acid	5-hydroxy-2-oxopentanoic acid dehydrogenase	ADH2 yqhD 4hbd
10	1.1.1.c	alpha-ketoglutaryl-CoA	5-hydroxy-2-oxopentanoic acid	alpha-ketoglutaryl-CoA reductase (alcohol forming)	adhE2 mcr FAR
10	4.1.1.a	5-hydroxy-2-oxopentanoic acid	4-hydroxybutanal	5-hydroxy-2-oxopentanoic acid decarboxylase	pdc mdlC pdc1
10	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2 yqhD 4hbd
10	1.2.1.c	5-hydroxy-2-oxopentanoic acid	4-hydroxybutyryl-CoA	5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)	sucA, sucB, lpd bfmBB, bfmBAA, bfmBAB, bfmBAB, pdhD Bckdha, Bckdhb, Dbt, Dld

FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
10	2.7.2.a	NP_416799.1	*Escherichia coli*	acetate, propionate
		NP_349675	*Clostridium acetobutylicum*	butyrate
		NP_414777.1	*Escherichia coli*	glutamate
10	1.2.1.d	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
		NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
		P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate

TABLE 20-continued

BDO pathway from alpha-ketoglutarate.

10	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
10	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
10	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
10	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
10	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
		BAA03892.1	*Pseudomonas* sp	Acetaldehyde, Propionaldehyde, Butyraldehyde, Isobutyraldehyde and Formaldehyde
10	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
10	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
10	4.1.1.a	P06672.1	*Zymomonas mobilus*	2-oxopentanoic acid
		P20906.2	*Pseudomonas putida*	2-oxopentanoic acid
		P06169	*Saccharomyces cerevisiae*	pyruvate
10	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
10	1.2.1.c	NP_415254.1, NP_415255.1, NP_414658.1	*Escherichia coli*	Alpha-ketoglutarate
		NP_390283.1, NP_390285.1, NP_390284.1, P21880.1	*Bacillus subtilis*	2-keto acids derivatives of valine, leucine and isoleucine
		NP_036914.1, NP_062140.1, NP_445764.1, NP_955417.1	*Rattus norvegicus*	2-keto acids derivatives of valine, leucine and isoleucine

EXAMPLE IX

Exemplary BDO Pathways from Glutamate

This example describes exemplary BDO pathways from glutamate.

FIG. **11** depicts exemplary BDO pathways in which glutamate is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 21, along with exemplary genes encoding these enzymes.

Briefly, glutamate can be converted to glutamyl-CoA by glutamate CoA transferase (EC 2.8.3.a), glutamyl-CoA hydrolase (EC 3.1.2.a) or glutamyl-CoA ligase (or glutamyl-CoA synthetase) (EC 6.2.1.a). Alternatively, glutamate can be converted to glutamate-5-phosphate by glutamate 5-kinase (EC 2.7.2.a). Glutamate-5-phosphate can be converted to glutamate-5-semialdehyde by glutamate-5-semialdehyde dehydrogenase (phosphorylating) (EC 1.2.1.d). Glutamyl-CoA can be converted to glutamate-5-semialdehyde by glutamyl-CoA reductase (or glutamate-5-semialdehyde dehydrogenase) (EC 1.2.1.b). Glutamate-5-semialdehyde can be converted to 2-amino-5-hydroxypentanoic acid by glutamate-5-semialdehyde reductase (EC 1.1.1.a). Alternatively, glutamyl-CoA can be converted to 2-amino-5-hydroxypentanoic acid by glutamyl-CoA reductase (alcohol forming) (EC 1.1.1.c). 2-Amino-5-hydroxypentanoic acid can be converted to 5-hydroxy-2-oxopentanoic acid by 2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating) (EC 1.4.1.a) or 2-amino-5-hydroxypentanoic acid transaminase (EC 2.6.1.a). 5-Hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutanal by 5-hydroxy-2-oxopentanoic acid decarboxylase (EC 4.1.1.a). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a). Alternatively, 5-hydroxy-2-oxopentanoic acid can be converted to 4-hydroxybutyryl-CoA by 5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation) (EC 1.2.1.c).

TABLE 21

BDO pathway from glutamate.

FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
11	2.8.3.a	glutamate	glutamyl-CoA	glutamate CoA transferase	cat1, cat2, cat3 gctA, gctB atoA, atoD
11	3.1.2.a	glutamate	glutamyl-CoA	glutamyl-CoA hydrolase	tesB acot12 hibch
11	6.2.1.a	glutamate	glutamyl-CoA	glutamyl-CoA ligase (or glutamyl-CoA synthetase)	sucCD phl bioW
11	2.7.2.a	glutamate	glutamate-5-phosphate	glutamate 5-kinase	ackA buk1 proB
11	1.2.1.d	glutamate-5-phosphate	glutamate-5-semialdehyde	glutamate-5-semialdehyde dehydrogenase (phosphorylating)	proA asd gapA
11	1.2.1.b	glutamyl-CoA	glutamate-5-semialdehyde	glutamyl-CoA reductase (or glutamate-5-semialdehyde dehydrogenase)	sucD Msed_0709 bphG
11	1.1.1.a	glutamate-5-semialdehyde	2-amino-5-hydroxypentanoic acid	glutamate-5-semialdehyde reductase	ADH2 yqhD 4hbd
11	1.1.1.c	glutamyl-CoA	2-amino-5-hydroxypentanoic acid	glutamyl-CoA reductase (alcohol forming)	adhE2 mcr FAR
11	1.4.1.a	2-amino-5-hydroxypentanoic acid	5-hydroxy-2-oxopentanoic acid	2-amino-5-hydroxypentanoic acid oxidoreductase (deaminating)	gdhA ldh nadX
11	2.6.1.a	2-amino-5-hydroxypentanoic acid	5-hydroxy-2-oxopentanoic acid	2-amino-5-hydroxypentanoic acid transaminase	aspC AAT2 avtA
11	4.1.1.a	5-hydroxy-2-oxopentanoic acid	4-hydroxybutanal	5-hydroxy-2-oxopentanoic acid decarboxylase	pdc mdlC pdc1
11	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2 yqhD 4hbd
11	1.2.1.c	5-hydroxy-2-oxopentanoic acid	4-hydroxybutyryl-CoA	5-hydroxy-2-oxopentanoic acid dehydrogenase (decarboxylation)	sucA, sucB, lpd bfmBB, bfmBAA, bfmBAB, bfmBAB, pdhD Bckdha, Bckdhb, Dbt, Dld

TABLE 21-continued

BDO pathway from glutamate.

FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
11	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
11	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
11	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
11	2.7.2.a	NP_416799.1	*Escherichia coli*	acetate, propionate
		NP_349675	*Clostridium acetobutylicum*	butyrate
		NP_414777.1	*Escherichia coli*	glutamate
11	1.2.1.d	NP_414778.1	*Escherichia coli*	L-glutamyl-5-phospate
		NP_417891.1	*Escherichia coli*	L-4-aspartyl-phosphate
		P0A9B2.2	*Escherichia coli*	Glyceraldehyde-3-phosphate
11	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
		BAA03892.1	*Pseudomonas* sp	Acetaldehyde, Propionaldehyde, Butyraldehyde, Isobutyraldehyde and Formaldehyde
11	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
11	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
11	1.4.1.a	P00370	*Escherichia coli*	glutamate
		P0A393	*Bacillus cereus*	leucine, isoleucine, valine, 2-aminobutanoate
		NP_229443.1	*Thermotoga maritima*	aspartate
11	2.6.1.a	NP_415448.1	*Escherichia coli*	aspartate
		P23542.3	*Saccharomyces cerevisiae*	aspartate
		YP_026231.1	*Escherichia coli*	valine, alpha-aminobutyrate
11	4.1.1.a	P06672.1	*Zymomonas mobilus*	2-oxopentanoic acid
		P20906.2	*Pseudomonas putida*	2-oxopentanoic acid
		P06169	*Saccharomyces cerevisiae*	pyruvate
11	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde
11	1.2.1.c	NP_415254.1, NP_415255.1, NP_414658.1	*Escherichia coli*	Alpha-ketoglutarate
		NP_390283.1, NP_390285.1, NP_390284.1, P21880.1	*Bacillus subtilis*	2-keto acids derivatives of valine, leucine and isoleucine

TABLE 21-continued

BDO pathway from glutamate.		
NP_036914.1, NP_062140.1, NP_445764.1, NP_955417.1	*Rattus norvegicus*	2-keto acids derivatives of valine, leucine and isoleucine

EXAMPLE X

Exemplary BDO from Acetoacetyl-CoA

This example describes an exemplary BDO pathway from acetoacetyl-CoA.

FIG. **12** depicts exemplary BDO pathways in which acetoacetyl-CoA is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 22, along with exemplary genes encoding these enzymes.

Briefly, acetoacetyl-CoA can be converted to 3-hydroxybutyryl-CoA by 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.a). 3-Hydroxybutyryl-CoA can be converted to crotonoyl-CoA by 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.a). Crotonoyl-CoA can be converted to vinylacetyl-CoA by vinylacetyl-CoA A-isomerase (EC 5.3.3.3). Vinylacetyl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA dehydratase (EC 4.2.1.a). 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 22

BDO pathway from acetoacetyl-CoA.					
FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
12	1.1.1.a	acetoacetyl-CoA	3-hydroxybutyryl-CoA	3-hydroxybutyryl-CoA dehydrogenase	hbd
					hbd Msed_1423
12	4.2.1.a	3-hydroxybutyryl-CoA	crotonoyl-CoA	3-hydroxybutyryl-CoA dehydratase	crt
					maoC paaF
12	5.3.3.3	crotonoyl-CoA	vinylacetyl-CoA	vinylacetyl-CoA Δ-isomerase	abfD
					abfD abfD
12	4.2.1.a	vinylacetyl-CoA	4-hydroxybutyryl-CoA	4-hydroxybutyryl-CoA dehydratase	abfD
					abfD abfD
12	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	adhE2
					mcr FAR
12	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	sucD
					sucD Msed_0709
12	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2
					yqhD 4hbd

FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
12	1.1.1.a	NP_349314.1	*Clostridium acetobutylicum*	3-hydroxybutyryl-CoA
		AAM14586.1	*Clostridium beijerinckii*	3-hydroxybutyryl-CoA
		YP_001191505	*Metallosphaera sedula*	presumed 3-hydroxybutyryl-CoA

TABLE 22-continued

BDO pathway from acetoacetyl-CoA.				
12	4.2.1.a	NP_349318.1	*Clostridium acetobutylicum*	3-hydroxybutyryl-CoA
		NP_415905.1	*Escherichia coli*	3-hydroxybutyryl-CoA
		NP_415911.1	*Escherichia coli*	3-hydroxyadipyl-CoA
12	5.3.3.3	YP_001396399.1	*Clostridium kluyveri* DSM 555	4-hydroxybutyryl-CoA
		P55792	*Clostridium aminobutyricum*	4-hydroxybutyryl-CoA
		YP_001928843	*Porphyromonas gingivalis* ATCC 33277	4-hydroxybutyryl-CoA
12	4.2.1.a	YP_001396399.1	*Clostridium kluyveri* DSM 555	4-hydroxybutyryl-CoA
		P55792	*Clostridium aminobutyricum*	4-hydroxybutyryl-CoA
		YP_001928843	*Porphyromonas gingivalis* ATCC 33277	4-hydroxybutyryl-CoA
12	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
12	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
12	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

EXAMPLE XI

Exemplary BDO Pathway from Homoserine

This example describes an exemplary BDO pathway from homoserine.

FIG. **13** depicts exemplary BDO pathways in which homoserine is converted to BDO. Enzymes of such an exemplary BDO pathway are listed in Table 23, along with exemplary genes encoding these enzymes.

Briefly, homoserine can be converted to 4-hydroxybut-2-enoate by homoserine deaminase (EC 4.3.1.a). Alternatively, homoserine can be converted to homoserine-CoA by homoserine CoA transferase (EC 2.8.3.a), homoserine-CoA hydrolase (EC 3.1.2.a) or homoserine-CoA ligase (or homoserine-CoA synthetase) (EC 6.2.1.a). Homoserine-CoA can be converted to 4-hydroxybut-2-enoyl-CoA by homoserine-CoA deaminase (EC 4.3.1.a). 4-Hydroxybut-2-enoate can be converted to 4-hydroxybut-2-enoyl-CoA by 4-hydroxybut-2-enoyl-CoA transferase (EC 2.8.3.a), 4-hydroxybut-2-enoyl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybut-2-enoyl-CoA ligase (or 4-hydroxybut-2-enoyl-CoA synthetase) (EC 6.2.1.a). Alternatively, 4-hydroxybut-2-enoate can be converted to 4-hydroxybutyrate by 4-hydroxybut-2-enoate reductase (EC 1.3.1.a). 4-Hydroxybutyrate can be converted to 4-hydroxybutyryl-coA by 4-hydroxybutyryl-CoA transferase (EC 2.8.3.a), 4-hydroxybutyryl-CoA hydrolase (EC 3.1.2.a), or 4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase) (EC 6.2.1.a). 4-Hydroxybut-2-enoyl-CoA can be converted to 4-hydroxybutyryl-CoA by 4-hydroxybut-2-enoyl-CoA reductase (EC 1.3.1.a). 4-Hydroxybutyryl-CoA can be converted to 1,4-butanediol by 4-hydroxybutyryl-CoA reductase (alcohol forming) (EC 1.1.1.c). Alternatively, 4-hydroxybutyryl-CoA can be converted to 4-hydroxybutanal by 4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase) (EC 1.2.1.b). 4-Hydroxybutanal can be converted to 1,4-butanediol by 1,4-butanediol dehydrogenase (EC 1.1.1.a).

TABLE 23

BDO pathway from homoserine.					
FIG.	EC class	Desired substrate	Desired product	Enzyme name	Gene name
13	4.3.1.a	homoserine	4-hydroxybut-2-enoate	homoserine deaminase	aspA aspA aspA
13	2.8.3.a	homoserine	homoserine-CoA	homoserine CoA transferase	cat1, cat2, cat3 gctA, gctB atoA, atoD

TABLE 23-continued

BDO pathway from homoserine.

13	3.1.2.a	homoserine	homoserine-CoA	homoserine-CoA hydrolase	tesB acot12 hibch
13	6.2.1.a	homoserine	homoserine-CoA	homoserine-CoA ligase (or homoserine-CoA synthetase)	sucCD phl bioW
13	4.3.1.a	homoserine-CoA	4-hydroxybut-2-enoyl-CoA	homoserine-CoA deaminase	acl1 acl2 MXAN_4385
13	2.8.3.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA transferase	cat1, cat2, cat3 gctA, gctB atoA, atoD
13	3.1.2.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA hydrolase	tesB acot12 hibch
13	6.2.1.a	4-hydroxybut-2-enoate	4-hydroxybut-2-enoyl-CoA	4-hydroxybut-2-enoyl-CoA ligase (or 4-hydroxybut-2-enoyl-CoA synthetase)	sucCD phl bioW
13	1.3.1.a	4-hydroxybut-2-enoate	4-hydroxybutyrate	4-hydroxybut-2-enoate reductase	enr enr enr
13	2.8.3.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA transferase	cat1, cat2, cat3 gctA, gctB atoA, atoD
13	3.1.2.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA hydrolase	tesB acot12 hibch
13	6.2.1.a	4-hydroxybutyrate	4-hydroxybutyryl-coA	4-hydroxybutyryl-CoA ligase (or 4-hydroxybutyryl-CoA synthetase)	sucCD phl bioW
13	1.3.1.a	4-hydroxybut-2-enoyl-CoA	4-hydroxybutyryl-CoA	4-hydroxybut-2-enoyl-CoA reductase	bcd, etfA, etfB TER TDE0597
8	1.1.1.c	4-hydroxybutyryl-CoA	1,4-butanediol	4-hydroxybutyryl-CoA reductase (alcohol forming)	adhE2 mcr FAR
8	1.2.1.b	4-hydroxybutyryl-CoA	4-hydroxybutanal	4-hydroxybutyryl-CoA reductase (or 4-hydroxybutanal dehydrogenase)	sucD sucD Msed_0709
8	1.1.1.a	4-hydroxybutanal	1,4-butanediol	1,4-butanediol dehydrogenase	ADH2 yqhD 4hbd

TABLE 23-continued

BDO pathway from homoserine.

FIG.	EC class	GenBank ID (if available)	*Organism*	Known Substrate
13	4.3.1.a	NP_418562	*Escherichia coli*	aspartate
		P44324.1	*Haemophilus influenzae*	aspartate
		P07346	*Pseudomonas fluorescens*	aspartate
13	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
13	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
13	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
13	4.3.1.a	CAG29274.1	*Clostridium propionicum*	beta-alanyl-CoA
		CAG29275.1	*Clostridium propionicum*	beta-alanyl-CoA
		YP_632558.1	*Myxococcus xanthus*	beta-alanyl-CoA
13	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
13	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
13	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
13	1.3.1.a	CAA71086.1	*Clostridium tyrobutyricum*	
		CAA76083.1	*Clostridium kluyveri*	
		YP_430895.1	*Moorella thermoacetica*	
13	2.8.3.a	P38946.1, P38942.2, EDK35586.1	*Clostridium kluyveri*	succinate, 4-hydroxybutyrate, butyrate
		CAA57199.1, CAA57200.1	*Acidaminococcus fermentans*	glutarate
		P76459.1, P76458.1	*Escherichia coli*	butanoate
13	3.1.2.a	NP_414986	*Escherichia coli*	adipyl-CoA
		NP_570103.1	*Rattus norvegicus*	butyryl-CoA
		Q6NVY1.2	*Homo sapiens*	3-hydroxypropanoyl-CoA
13	6.2.1.a	NP_415256.1, AAC73823.1	*Escherichia coli*	succinate
		CAJ15517.1	*Penicillium chrysogenum*	phenylacetate
		NP_390902.2	*Bacillus subtilis*	6-carboxyhexanoate
13	1.3.1.a	NP_349317.1, NP_349315.1, NP_349316.1	*Clostridium acetobutylicum*	
		Q5EU90.1	*Euglena gracilis*	
		NP_971211.1	*Treponema denticola*	

TABLE 23-continued

BDO pathway from homoserine.				
8	1.1.1.c	AAK09379.1	*Clostridium acetobutylicum*	butanoyl-CoA
		AAS20429.1	*Chloroflexus aurantiacus*	malonyl-CoA
		AAD38039.1	*Simmondsia chinensis*	long chain acyl-CoA
8	1.2.1.b	P38947.1	*Clostridium kluyveri*	Succinyl-CoA
		NP_904963.1	*Porphyromonas gingivalis*	Succinyl-CoA
		YP_001190808.1	*Metallosphaera sedula*	Malonyl-CoA
8	1.1.1.a	NP_014032.1	*Saccharymyces cerevisiae*	general
		NP_417484.1	*Escherichia coli*	>C3
		L21902.1	*Clostridium kluyveri* DSM 555	Succinate semialdehyde

EXAMPLE XII

BDO Producing Strains Expressing Succinyl-CoA Synthetase

This example describes increased production of BDO in BDO producing strains expressing succinyl-CoA synthetase.

As discussed above, succinate can be a precursor for production of BDO by conversion to succinyl-CoA (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Therefore, the host strain was genetically modified to overexpress the *E. coli* sucCD genes, which encode succinyl-CoA synthetase. The nucleotide sequence of the *E. coli* sucCD operon is shown in FIG. **14**A, and the amino acid sequences for the encoded succinyl-CoA synthetase subunits are shown in FIGS. **14**B and **14**C. Briefly, the *E. coli* sucCD genes were cloned by PCR from *E. coli* chromosomal DNA and introduced into multicopy plasmids pZS*13, pZA13, and pZE33 behind the PA1lacO-1 promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)) using standard molecular biology procedures.

The *E. coli* sucCD genes, which encode the succinyl-CoA synthetase, were overexpressed. The results showed that introducing into the strains sucCD to express succinyl-CoA synthetase improved BDO production in various strains compared to either native levels of expression or expression of cat1, which is a succinyl-CoA/acetyl-CoA transferase. Thus, BDO production was improved by overexpressing the native *E. coli* sucCD genes encoding succinyl-CoA synthetase.

EXAMPLE XIII

Expression of Heterologous Genes Encoding BDO Pathway Enzymes

This example describes the expression of various non-native pathway enzymes to provide improved production of BDO.

Alpha-Ketoglutarate Decarboxylase.

The *Mycobacterium bovis* sucA gene encoding alpha-ketoglutarate decarboxylase was expressed in host strains. Overexpression of *M. bovis* sucA improved BDO production (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The nucleotide and amino acid sequences of *M. bovis* sucA and the encoded alpha-ketoglutarate decarboxylase are shown in FIG. **15**.

To construct the *M. bovis* sucA expressing strains, fragments of the sucA gene encoding the alpha-ketoglutarate decarboxylase were amplified from the genomic DNA of *Mycobacterium bovis* BCG (ATCC 19015; American Type Culture Collection, Manassas Va.) using primers shown below. The full-length gene was assembled by ligation reaction of the four amplified DNA fragments, and cloned into expression vectors pZS*13 and pZE23 behind the $P_{A1lacO-1}$ promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)). The nucleotide sequence of the assembled gene was verified by DNA sequencing.

```
Primers for fragment 1:
                                        (SEQ ID NO: 3)
5'-ATGTACCGCAAGTTCCGC-3'

                                        (SEQ ID NO: 4)
5'-CAATTTGCCGATGCCCAG-3'

Primers for fragment 2:
                                        (SEQ ID NO: 5)
5'-GCTGACCACTGAAGACTTTG-3'

                                        (SEQ ID NO: 6)
5'-GATCAGGGCTTCGGTGTAG-3'

Primers for fragment 3:
                                        (SEQ ID NO: 7)
5'-TTGGTGCGGGCCAAGCAGGATCTGCTC-3'

                                        (SEQ ID NO: 8)
5'-TCAGCCGAACGCCTCGTCGAGGATCTCCTG-3'

Primers for fragment 4:
                                        (SEQ ID NO: 9)
5'-TGGCCAACATAAGTTCACCATTCGGGCAAAAC-3'

                                       (SEQ ID NO: 10)
5'-TCTCTTCAACCAGCCATTCGTTTTGCCCG-3'
```

Functional expression of the alpha-ketoglutarate decarboxylase was demonstrated using both in vitro and in vivo assays. The SucA enzyme activity was measured by following a previously reported method (Tian et al., *Proc. Natl. Acad. Sci. USA* 102:10670-10675 (2005)). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 0.2 mM thiamine pyrophosphate, 1 mM $MgCl_2$, 0.8 mM ferricyanide, 1 mM alpha-ketoglutarate and cell crude lysate. The enzyme activity was monitored by the reduction of ferricyanide at 430 nm. The in vivo function of the SucA enzyme was verified using *E. coli* whole-cell culture. Single colonies of *E. coli* MG1655 $lacI^q$ transformed with plasmids encoding the SucA enzyme and the 4-hydroxybutyrate dehy-

drogenase (4Hbd) was inoculated into 5 mL of LB medium containing appropriate antibiotics. The cells were cultured at 37° C. overnight aerobically. A 200 uL of this overnight culture was introduced into 8 mL of M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4C1, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. Microaerobic conditions were established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with a 23G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. The protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the culture reached mid-log growth phase. As controls, *E. coli* MG1655 $lacI^q$ strains transformed with only the plasmid encoding the 4-hydroxybutyrate dehydrogenase and only the empty vectors were cultured under the same condition (see Table 23). The accumulation of 4-hydroxybutyrate (4HB) in the culture medium was monitored using LCMS method. Only the *E. coli* strain expressing the *Mycobacterium* alpha-ketoglutarate decarboxylase produced significant amount of 4HB (see FIG. **16**).

TABLE 24

Three strains containing various plasmid controls and encoding sucA and 4-hydroxybutyrate dehydrogenase.

	Host	pZE13	pZA33
1	MG1655 laclq	vector	vector
2	MG1655 laclq	vector	4hbd
3	MG1655 laclq	sucA	4hbd

A separate experiment demonstrated that the alpha-ketoglutarate decarboxylase pathway functions independently of the reductive TCA cycle. *E. coli* strain ECKh-401 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA) was used as the host strain (see Table 25). All the three constructs contained the gene encoding 4HB dehydrogenase (4Hbd). Construct 1 also contained the gene encoding the alpha-ketoglutarate decarboxylase (sucA). Construct 2 contained the genes encoding the succinyl-CoA synthetase (sucCD) and the CoA-dependent succinate semialdehyde dehydrogenase (sucD), which are required for the synthesis of 4HB via the reductive TCA cycle. Construct 3 contains all the genes from 1 and 2. The three *E. coli* strains were cultured under the same conditions as described above except the second culture was under the microaerobic condition. By expressing the SucA enzyme, construct 3 produced more 4HB than construct 2, which relies on the reductive TCA cycle for 4HB synthesis (see FIG. **17**).

Further support for the contribution of alpha-ketoglutarate decarboxylase to production of 4HB and BDO was provided by flux analysis experiments. Cultures of ECKh-432, which contains both sucCD-sucD and sucA on the chromosome, were grown in M9 minimal medium containing a mixture of 1-13C-glucose (60%) and U-13C-glucose (40%). The biomass was harvested, the protein isolated and hydrolyzed to amino acids, and the label distribution of the amino acids analyzed by gas chromatography-mass spectrometry (GCMS) as described previously (Fischer and Sauer, *Eur. J. Biochem.* 270:880-891 (2003)). In addition, the label distribution of the secreted 4HB and BDO was analyzed by GCMS as described in WO2008115840 A2. This data was used to calculate the intracellular flux distribution using established methods (Suthers et al., *Metab. Eng.* 9:387-405 (2007)). The results indicated that between 56% and 84% of the alpha-ketoglutarate was channeled through alpha-ketoglutarate decarboxylase into the BDO pathway. The remainder was oxidized by alpha-ketoglutarate dehydrogenase, which then entered BDO via the succinyl-CoA route.

These results demonstrate 4-hydroxybutyrate producing strains that contain the sucA gene from *Mycobacterium bovis* BCG expressed on a plasmid. When the plasmid encoding this gene is not present, 4-hydroxybutyrate production is negligible when sucD (CoA-dependant succinate semialdehyde dehydrogenase) is not expressed. The *M. bovis* gene is a close homolog of the *Mycobacterium tuberculosis* gene whose enzyme product has been previously characterized (Tian et al., supra, 2005).

Succinate Semialdehyde Dehydrogenase (CoA-Dependent), 4-Hydroxybutyrate Dehydrogenase, and 4-Hydroxybutyryl-CoA/Acetyl-CoA Transferase.

The genes from *Porphyromonas gingivalis* W83 can be effective components of the pathway for 1,4-butanediol production (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The nucleotide sequence of CoA-dependent succinate semialdehyde dehydrogenase (sucD) from *Porphyromonas gingivalis* is shown in FIG. **18**A, and the encoded amino acid sequence is shown in FIG. **18**B. The nucleotide sequence of 4-hydroxybutyrate dehydrogenase (4hbd) from *Porphyromonas gingivalis* is shown in FIG. **19**A, and the encoded amino acid sequence is shown in FIG. **19**B. The nucleotide sequence of 4-hydroxybutyrate CoA transferase (cat2) from *Porphyromonas gingivalis* is shown in FIG. **20**A, and the encoded amino acid sequence is shown in FIG. **20**B.

Briefly, the genes from *Porphyromonas gingivalis* W83 encoding succinate semialdehyde dehydrogenase (CoA-dependent) and 4-hydroxybutyrate dehydrogenase, and in some cases additionally 4-hydroxybutyryl-CoA/acetyl-CoA, were cloned by PCR from *P. gingivalis* chromosomal DNA and introduced into multicopy plasmids pZS*13, pZA13, and pZE33 behind the PA1lacO-1 promoter (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)) using standard molecular biology procedures. These plasmids were then introduced into host strains.

The *Porphyromonas gingivalis* W83 genes were introduced into production strains as described above. Some strains included only succinate semialdehyde dehydrogenase (CoA-dependant) and 4-hydroxybutyrate dehydrogenase without 4-hydroxybutyryl-CoA/acetyl-CoA transferase.

Butyrate Kinase and Phosphotransbutyrylase.

Butyrate kinase (BK) and phosphotransbutyrylase (PTB) enzymes can be utilized to produce 4-hydroxybutyryl-CoA (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). In particular, the *Clostridium acetobutylicum* genes, buk1 and ptb, can be utilized as part of a functional BDO pathway.

Initial experiments involved the cloning and expression of the native *C. acetobutylicum* PTB (020) and BK (021) genes in *E. coli*. Where required, the start codon and stop codon for each gene were modified to "ATG" and "TAA," respectively, for more optimal expression in *E. coli*. The *C. acetobutylicum* gene sequences (020N and 021N) and their corresponding translated peptide sequences are shown in FIGS. **21** and **22**.

The PTB and BK genes exist in *C. acetobutylicum* as an operon, with the PTB (020) gene expressed first. The two genes are connected by the sequence "atta aagttaagtg gag-

gaatgtt aac" (SEQ ID NO:11) that includes a re-initiation ribosomal binding site for the downstream BK (021) gene. The two genes in this context were fused to lac-controlled promoters in expression vectors for expression in *E. coli* (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)).

Expression of the two proteins from these vector constructs was found to be low in comparison with other exogenously expressed genes due to the high incidence of codons in the *C. acetobutylicum* genes that occur only rarely in *E. coli*. Therefore new 020 and 021 genes were predicted that changed rare codons for alternates that are more highly represented in *E. coli* gene sequences. This method of codon optimization followed algorithms described previously (Sivaraman et al., *Nucleic Acids Res.* 36:e16 (2008)). This method predicts codon replacements in context with their frequency of occurrence when flanked by certain codons on either side. Alternative gene sequences for 020 (FIG. **23**) and 021 (FIG. **24**) were determined in which increasing numbers of rare codons were replaced by more prevalent codons (A<B<C<D) based on their incidence in the neighboring codon context. No changes in actual peptide sequence compared to the native 020 and 021 peptide sequences were introduced in these predicted sequences.

The improvement in expression of the BK and PTB proteins resulting from codon optimization is shown in FIG. **25**A. Expression of the native gene sequences is shown in lane 2, while expression of the 020B-021B and 020C-021C is shown in lanes 3 and 4, respectively. Higher levels of protein expression in the codon-optimized operons 020B-021B (2021B) and 020C-021C (2021C) also resulted in increased activity compared to the native operon (2021n) in equivalently-expressed *E. coli* crude extracts (FIG. **25**B).

The codon optimized operons were expressed on a plasmid in strain ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd) along with the *C. acetobutylicum* aldehyde dehydrogenase to provide a complete BDO pathway. Cells were cultured in M9 minimal medium containing 20 g/L glucose, using a 23G needle to maintain microaerobic conditions as described above. The resulting conversion of glucose to the final product BDO was measured. Also measured was the accumulation of gamma-butyrolactone (GBL), which is a spontaneously rearranged molecule derived from 4Hb-CoA, the immediate product of the PTB-BK enzyme pair. FIG. **26** shows that expression of the native 2021n operon resulted in comparable BDO levels to an alternative enzyme function, Cat2 (034), that is capable of converting 4HB and free CoA to 4HB-CoA. GBL levels of 034 were significantly higher than 2021n, suggesting that the former enzyme has more activity than PTB-BK expressed from the native genes. However levels of both BDO and GBL were higher than either 034 or 2021n when the codon-optimized variants 2021B and 2021C were expressed, indicating that codon optimization of the genes for PTB and BK significantly increases their contributions to BDO synthesis in *E. coli*.

These results demonstrate that butyrate kinase (BK) and phosphotransbutyrylase (PTB) enzymes can be employed to convert 4-hydroxybutyrate to 4-hydroxybutyryl-CoA. This eliminates the need for a transferase enzyme such as 4-hydroxybutyryl-CoA/Acetyl-CoA transferase, which would generate one mole of acetate per mol of 4-hydroxybutyryl-CoA produced. The enzymes from *Clostridium acetobutylicum* are present in a number of engineered strains for BDO production.

4-hydroxybutyryl-CoA Reductase.

The *Clostridium beijerinckii* ald gene can be utilized as part of a functional BDO pathway (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). The *Clostridium beijerinckii* ald can also be utilized to lower ethanol production in BDO producing strains. Additionally, a specific codon-optimized ald variant (GNM0025B) was found to improve BDO production.

The native *C. beijerinckii* ald gene (025n) and the predicted protein sequence of the enzyme are shown in FIG. **27**. As was seen for the *Clostridium acetobutylicum* PTB and BK genes, expression of the native *C. beijerinckii* ald gene was very low in *E. coli*. Therefore, four codon-optimized variants for this gene were predicted. FIGS. **28**A-**28**D show alternative gene sequences for 025, in which increasing numbers of rare codons are replaced by more prevalent codons (A<B<C<D) based on their incidence in the neighboring codon context (25A, P=0.05; 25B, P=0.1; 25C, P=0.15; 25D, P=1). No changes in actual peptide sequence compared to the native 025 peptide sequence were introduced in these predictions. Codon optimization significantly increased expression of the *C. beijerinckii* ald (see FIG. **29**), which resulted in significantly higher conversion of glucose to BDO in cells expressing the entire BDO pathway (FIG. **30**A).

The native and codon-optimized genes were expressed on a plasmid along with *P. gingivalis* Cat2, in the host strain ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd), thus containing a complete BDO pathway. Cells were cultured microaerobically in M9 minimal medium containing 20 g/L glucose as described above. The relative production of BDO and ethanol by the *C. beijerinckii* Ald enzyme (expressed from codon-optimized variant gene 025B) was compared with the *C. acetobutylicum* AdhE2 enzyme (see FIG. **30**B). The *C. acetobutylicum* AdhE2 enzyme (002C) produced nearly 4 times more ethanol than BDO. In comparison, the *C. beijerinckii* Ald (025B) (in conjunction with an endogenous ADH activity) produced equivalent amounts of BDO, yet the ratio of BDO to ethanol production was reversed for this enzyme compared to 002C. This suggests that the *C. beijerinckii* Ald is more specific for 4HB-CoA over acetyl-coA than the *C. acetobutylicum* AdhE2, and therefore the former is the preferred enzyme for inclusion in the BDO pathway.

The *Clostridium beijerinckii* ald gene (Toth et al., *Appl. Environ. Microbiol.* 65:4973-4980 (1999)) was tested as a candidate for catalyzing the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutanal. Over fifty aldehyde dehydrogenases were screened for their ability to catalyze the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutyraldehyde. The *C. beijerinckii* ald gene was chosen for implementation into BDO-producing strains due to the preference of this enzyme for 4-hydroxybutyryl-CoA as a substrate as opposed to acetyl-CoA. This is important because most other enzymes with aldehyde dehydrogenase functionality (for example, adhE2 from *C. acetobutylicum* (Fontaine et al., J Bacteriol. 184:821-830 (2002)) preferentially convert acetyl-CoA to acetaldehyde, which in turn is converted to ethanol. Utilization of the *C. beijerinckii* gene lowers the amount of ethanol produced as a byproduct in BDO-producing organisms. Also, a codon-optimized version of this gene expresses very well in *E. coli* (Sivaraman et al., *Nucleic Acids Res.* 36:e16 (2008)).

4-hydroxybutanal Reductase.

4-hydroxybutanal reductase activity of adh1 from *Geobacillus thermoglucosidasius* (M10EXG) was utilized. This led to improved BDO production by increasing 4-hydroxybutanal reductase activity over endogenous levels.

Multiple alcohol dehydrogenases were screened for their ability to catalyze the reduction of 4-hydroxybutanal to BDO. Most alcohol dehydrogenases with high activity on butyraldehyde exhibited far lower activity on 4-hydroxybutyraldehyde. One notable exception is the adh1 gene from *Geobacillus thermoglucosidasius* M10EXG (Jeon et al., *J. Biotechnol.* 135:127-133 (2008)) (GNM0084), which exhibits high activity on both 4-hydroxybutanal and butanal.

The native gene sequence and encoded protein sequence if the adh1 gene from *Geobacillus thermoglucosidasius* are shown in FIG. **31**. The *G. thermoglucosidasius* ald1 gene was expressed in *E. coli*.

The Adh1 enzyme (084) expressed very well from its native gene in *E. coli* (see FIG. **32**A). In ADH enzyme assays, the *E. coli* expressed enzyme showed very high reductive activity when butyraldehyde or 4HB-aldehyde were used as the substrates (see FIG. **32**B). The Km values determined for these substrates were 1.2 mM and 4.0 mM, respectively. These activity values showed that the Adh1 enzyme was the most active on reduction of 4HB-aldehyde of all the candidates tested.

The 084 enzyme was tested for its ability to boost BDO production when coupled with the *C. beijerinckii* ald. The 084 gene was inserted behind the *C. beijerinckii* ald variant 025B gene to create a synthetic operon that results in coupled expression of both genes. Similar constructs linked 025B with other ADH candidate genes, and the effect of including each ADH with 025B on BDO production was tested. The host strain used was ECKh-459 (ΔadhE ldhA ΔpflB ΔlpdA::fnr-pflB6-K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd fimD:: *C. acetobutylicum buk*1, *C. acetobutylicum* ptb), which contains the remainder of the BDO pathway on the chromosome. The 084 ADH expressed in conjunction with 025B showed the highest amount of BDO (right arrow in FIG. **33**) when compared with 025B only (left arrow in FIG. **33**) and in conjunction with endogenous ADH functions. It also produced more BDO than did other ADH enzymes when paired with 025B, indicated as follows: 026A-C, codon-optimized variants of *Clostridium acetobutylicum* butanol dehydrogenase; 050, *Zymomonas mobilis* alcohol dehydrogenase I; 052, *Citrobacter freundii* 1,3-propanediol dehydrogenase; 053, *Lactobacillus brevis* 1,3-propanediol dehydrogenase; 057, *Bacteroides fragilis* lactaldehyde reductase; 058, *E. coli* 1,3-propanediol dehydrogenase; 071, *Bacillus subtilis* 168 alpha-ketoglutarate semialdehyde dehydrogenase. The constructs labeled "PT5lacO" are those in which the genes are driven by the PT5lacO promoter. In all other cases, the PA1lacO-1 promoter was used. This shows that inclusion of the 084 ADH in the BDO pathway increased BDO production.

EXAMPLE XIV

BDO Producing Strains Expressing Pyruvate Dehydrogenase

This example describes the utilization of pyruvate dehydrogenase (PDH) to enhance BDO production. Heterologous expression of the *Klebsiella pneumonia* lpdA gene was used to enhance BDO production.

Computationally, the NADH-generating conversion of pyruvate to acetyl-CoA is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351; WO 2008/018930; Kim et al., *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim et al., *J. Bacteriol.* 190:3851-3858 (2008); Menzel et al., *J. Biotechnol.* 56:135-142 (1997)). Lack of PDH activity was shown to reduce the maximum anaerobic theoretical yield of BDO by 11% if phosphoenolpyruvate carboxykinase (PEPCK) activity cannot be attained and by 3% if PEPCK activity can be attained. More importantly, however, absence of PDH activity in the OptKnock strain #439, described in WO 2009/023493 and U.S. publication 2009/0047719, which has the knockout of ADHEr, ASPT, LDH_D, MDH and PFLi, would reduce the maximum anaerobic yield of BDO by 54% or by 43% if PEPCK activity is absent or present, respectively. In the presence of an external electron acceptor, lack of PDH activity would reduce the maximum yield of the knockout strain by 10% or by 3% assuming that PEPCK activity is absent or present, respectively.

PDH is one of the most complicated enzymes of central metabolism and is comprised of 24 copies of pyruvate decarboxylase (E1) and 12 molecules of dihydrolipoyl dehydrogenase (E3), which bind to the outside of the dihydrolipoyl transacetylase (E2) core. PDH is inhibited by high NADH/NAD, ATP/ADP, and Acetyl-CoA/CoA ratios. The enzyme naturally exhibits very low activity under oxygen-limited or anaerobic conditions in organisms such as *E. coli* due in large part to the NADH sensitivity of E3, encoded by lpdA. To this end, an NADH-insensitive version of the lpdA gene from *Klebsiella pneumonia* was cloned and expressed to increase the activity of PDH under conditions where the NADH/NAD ratio is expected to be high.

Replacement of the Native lpdA.

The pyruvate dehydrogenase operon of *Klebsiella pneumoniae* is between 78 and 95% identical at the nucleotide level to the equivalent operon of *E. coli*. It was shown previously that *K. pneumoniae* has the ability to grow anaerobically in presence of glycerol (Menzel et al., *J. Biotechnol.* 56:135-142 (1997); Menzel et al., *Biotechnol. Bioeng.* 60:617-626 (1998)). It has also been shown that two mutations in the lpdA gene of the operon of *E. coli* would increase its ability to grow anaerobically (Kim et al. *Appl. Environ. Microbiol.* 73:1766-1771 (2007); Kim et al., *J. Bacteriol.* 190:3851-3858 (2008)). The lpdA gene of *K. pneumonia* was amplified by PCR using genomic DNA (ATCC700721D) as template and the primers KP-lpdA-Bam (5'-acacgcggatccaacgtcccgg-3') (SEQ ID NO:12) and KP-lpdA-Nhe (5'-agcggctccgctagccgcttatg-3') (SEQ ID NO:13). The resulting fragment was cloned into the vector pCR-BluntII-TOPO (Invitrogen; Carlsbad Calif.), leading to plasmid pCR-KP-lpdA.

The chromosomal gene replacement was performed using a non-replicative plasmid and the sacB gene from *Bacillus subtilis* as a means of counterselection (Gay et al., *J. Bacteriol.* 153:1424-1431 (1983)). The vector used is pRE118 (ATCC87693) deleted of the oriT and IS sequences, which is 3.6 kb in size and carrying the kanamycin resistance gene. The sequence was confirmed, and the vector was called pRE118-V2 (see FIG. **34**).

The *E. coli* fragments flanking the lpdA gene were amplified by PCR using the combination of primers: EC-aceF-Pst (5'-aagccgttgctgcagctcttgagc-3') (SEQ ID NO:14)+EC-aceF-Bam2 (5'-atctccggcggtcggatccgtcg-3') (SEQ ID NO:15) and EC-yacH-Nhe (5'-aaagcggctagccacgccgc-3') (SEQ ID NO:16)+EC-yacH-Kpn (5'-attacacgaggtacccaacg-

3') (SEQ ID NO:17). A BamHI-XbaI fragment containing the lpdA gene of *K. pneumonia* was isolated from plasmid pCR-KP-lpdA and was then ligated to the above *E. coli* fragments digested with PstI+BamHI and NheI-KpnI respectively, and the pRE118-V2 plasmid digested with KpnI and PstI. The resulting plasmid (called pRE118-M2.1 lpdA yac) was subjected to Site Directed Mutagenesis (SDM) using the combination of primers KP-lpdA-HisTyr-F (5'-atgctggcgtacaaaggtgtcc-3') (SEQ ID NO:18) and (5'-ggacacctttgtacgccagcat-3') (SEQ ID NO:19) for the mutation of the His 322 residue to a Tyr residue or primers KP-lpdA-GluLys-F (5'-atcgcctacactaaaccagaagtgg-3') (SEQ ID NO:20) and KP-lpdA-GluLys-R (5'-ccacttctggtttagtgtaggcgat-3') (SEQ ID NO:21) for the mutation of the residue Glu 354 to Lys residue. PCR was performed with the Polymerase Pfu Turbo (Stratagene; San Diego Calif.). The sequence of the entire fragment as well as the presence of only the desired mutations was verified. The resulting plasmid was introduced into electro competent cells of *E. coli* ΔadhE::Frt-ΔldhA::Frt by transformation. The first integration event in the chromosome was selected on LB agar plates containing Kanamycin (25 or 50 mg/L). Correct insertions were verified by PCR using 2 primers, one located outside the region of insertion and one in the kanamycin gene (5'-aggcagttccataggatggc-3') (SEQ ID NO:22). Clones with the correct insertion were selected for resolution. They were sub-cultured twice in plain liquid LB at the desired temperature and serial dilutions were plated on LB-no salt-sucrose 10% plates. Clones that grew on sucrose containing plates were screened for the loss of the kanamycin resistance gene on LB-low salt agar medium and the lpdA gene replacement was verified by PCR and sequencing of the encompassing region. Sequence of the insertion region was verified, and is as described below. One clone (named 4-4-P1) with mutation Glu354Lys was selected. This clone was then transduced with P1 lysate of *E. coli* ΔPflB::Frt leading to strain ECKh-138 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322).

The sequence of the ECKh-138 region encompassing the aceF and lpdA genes is shown in FIG. **35**. The *K. pneumonia lpdA gene is underlined, and the codon changed in the Glu*354Lys mutant shaded. The protein sequence comparison of the native *E. coli* lpdA and the mutant *K. pneumonia* lpdA is shown in FIG. **36**.

To evaluate the benefit of using *K. pneumoniae* lpdA in a BDO production strain, the host strains AB3 and ECKh-138 were transformed with plasmids expressing the entire BDO pathway from strong, inducible promoters. Specifically, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd were expressed on the medium copy plasmid pZA33, and *P. gingivalis* Cat2 and *C. acetobutylicum* AdhE2 were expressed on the high copy plasmid pZE13. These plasmids have been described in the literature (Lutz and H. Bujard, *Nucleic Acids Res* 25:1203-1210 (1997)), and their use for BDO pathway expression is described in Example XIII and WO2008/115840.

Cells were grown anaerobically at 37° C. in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4C1, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 20 g/L glucose, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) to improve the buffering capacity, 10 μg/mL thiamine, and the appropriate antibiotics. Microaerobic conditions were established by initially flushing capped anaerobic bottles with nitrogen for 5 minutes, then piercing the septum with a 23G needle following inoculation. The needle was kept in the bottle during growth to allow a small amount of air to enter the bottles. 0.25 mM IPTG was added when OD600 reached approximately 0.2 to induce the pathway genes, and samples taken for analysis every 24 hours following induction. The culture supernatants were analyzed for BDO, 4HB, and other byproducts as described in Example II and in WO2008/115840. BDO and 4HB production in ECKh-138 was significantly higher after 48 hours than in AB3 or the host used in previous work, MG1655 ΔldhA (FIG. **37**).

PDH Promoter Replacement.

It was previously shown that the replacement of the pdhR repressor by a transcriptional fusion containing the Fnr binding site, one of the pflB promoters, and its ribosome binding site (RBS), thus leading to expression of the aceEF-lpd operon by an anaerobic promoter, should increase pdh activity anaerobically (Zhou et al., *Biotechnol. Lett.* 30:335-342 (2008)). A fusion containing the Fnr binding site, the pflB-p6 promoter and an RBS binding site were constructed by overlapping PCR. Two fragments were amplified, one using the primers aceE-upstream-RC (5'-tgacatgtaacacctac-cttctgtgcctgtgccagtggttgctgtgatatagaag-3') (SEQ ID NO:23) and pflBp6-Up-Nde (5'-ataataatacatatgaaccatgcgagttacgggc-ctataagccaggcg-3') (SEQ ID NO:24) and the other using primers aceE-EcoRV-EC (5'-agtttttcgatatctgcatcagacaccg-gcacattgaaacgg-3') (SEQ ID NO:25) and aceE-upstream (5'-ctggcacaggcacagaaggtaggtgttacatgtcagaacgtttacacaat-gacgtggatc-3') (SEQ ID NO:26). The tw fragments were assembled by overlapping PCR, and the final DNA fragment was digested with the restriction enzymes NdeI and BamHI. This fragment was subsequently introduced upstream of the aceE gene of the *E. coli* operon using pRE118-V2 as described above. The replacement was done in strains ECKh-138 and ECKh-422. The nucleotide sequence encompassing the 5' region of the aceE gene was verified and is shown in FIG. **37**. FIG. **37** shows the nucleotide sequence of 5' end of the aceE gene fused to the pflB-p6 promoter and ribosome binding site (RBS). The 5' italicized sequence shows the start of the aroP gene, which is transcribed in the opposite direction from the pdh operon. The 3' italicized sequence shows the start of the aceE gene. In upper case: pflB RBS. Underlined: FNR binding site. In bold: pflB-p6 promoter sequence.

lpdA Promoter Replacement.

The promoter region containing the fnr binding site, the pflB-p6 promoter and the RBS of the pflB gene was amplified by PCR using chromosomal DNA template and primers aceF-pflBp6-fwd (5'-agacaaatcggttgccgtttgttaagccaggcgagatatgatctatatc-3') (SEQ ID NO:27) and lpdA-RBS-B-rev (5'-gagttttgatttcagtactcatcatgtaacacctaccttcttgctgtgatatag-3') (SEQ ID NO:28). Plasmid 2-4a was amplified by PCR using primers B-RBS-lpdA fwd (5'-ctatatcacagcaagaaggtaggtgttacatgatgagtactgaaatcaaaactc-3') (SEQ ID NO:29) and pflBp6-aceF-rev (5'-gatatagatcatatctcgcctggcttaacaaacggcaaccgatttgtct-3') (SEQ ID NO:30). The two resulting fragments were assembled using the BPS cloning kit (BPS Bioscience; San Diego Calif.). The resulting construct was sequenced verified and introduced into strain ECKh-439 using the pRE118-V2 method described above. The nucleotide sequence encompassing the aceF-lpdA region in the resulting strain ECKh-456 is shown in FIG. **39**.

The host strain ECKh-439 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ackA fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd), the construction of which is described below, and the pdhR and lpdA promoter replacement derivatives ECKh-455 and ECKh-456, were tested for BDO production. The strains were transformed with pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald to

provide a complete BDO pathway. Cells were cultured in M9 minimal medium supplemented with 20 g/L glucose as described above. 48 hours after induction with 0.2 mM IPTG, the concentrations of BDO, 4HB, and pyruvate were as shown in FIG. **40**. The promoter replacement strains produce slightly more BDO than the isogenic parent.

These results demonstrated that expression of pyruvate dehydrogenase increased production of BDO in BDO producing strains.

EXAMPLE XV

BDO Producing Strains Expressing Citrate Synthase and Aconitase

This example describes increasing activity of citrate synthase and aconitase to increase production of BDO. An R163L mutation into gltA was found to improve BDO production. Additionally, an arcA knockout was used to improve BDO production.

Computationally, it was determined that flux through citrate synthase (CS) and aconitase (ACONT) is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Lack of CS or ACONT activity would reduce the maximum theoretical yield by 14% under anaerobic conditions. In the presence of an external electron acceptor, the maximum yield is reduced by 9% or by 6% without flux through CS or ACONT assuming the absence or presence of PEPCK activity, respectively. As with pyruvate dehydrogenase (PDH), the importance of CS and ACONT is greatly amplified in the knockout strain background in which ADHEr, ASPT, LDH_D, MDH and PFLi are knocked out (design #439)(see WO 2009/023493 and U.S. publication 2009/0047719, which is incorporated herein by reference).

The minimal OptKnock strain design described in WO 2009/023493 and U.S. publication 2009/0047719 had one additional deletion beyond ECKh-138, the mdh gene, encoding malate dehydrogenase. Deletion of this gene is intended to prevent flux to succinate via the reductive TCA cycle. The mdh deletion was performed using the λ red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)). The following oligonucleotides were used to PCR amplify the chloramphenicol resistance gene (CAT) flanked by FRT sites from pKD3:

(SEQ ID NO: 31)
S-mdh-Kan 5' - **TAT TGT GCA TAC AGA TGA ATT TTT ATG CAA ACA GTC AGC CCT**

GAA GAA GGG TGT AGG CTG GAG CTG CTT C - 3'

(SEQ ID NO: 32)
AS-mdh-Kan 5' - **CAA AAA ACC GGA GTC TGT GCT CCG GTT TTT TAT TAT CCG**

CTA ATC AAT TAC ATA TGA ATA TCC TCC TTA G - 3'.

Underlined regions indicate homology to pKD3 plasmid and bold sequence refers to sequence homology upstream and downstream of the mdh ORF. After purification, the PCR product was electroporated into ECKh-138 electrocompetent cells that had been transformed with pRedET (tet) and prepared according to the manufacturer's instructions (gen-ebridges.com/gb/pdf/K001%20Q%20E%20BAC%20 Modification%20Kit-version2.6-2007-screen.pdf). The PCR product was designed so that it integrated into the ECKh-138 genome at a region upstream of the mdh gene, as shown in FIG. **41**.

Recombinants were selected for chloramphenicol resistance and streak purified. Loss of the mdh gene and insertion of CAT was verified by diagnostic PCR. To remove the CAT gene, a temperature sensitive plasmid pCP20 containing a FLP recombinase (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)) was transformed into the cell at 30° C. and selected for ampicillin resistance (AMP). Transformants were grown nonselectively at 42° C. overnight to thermally induce FLP synthesis and to cause lose of the plasmid. The culture was then streak purified, and individual colonies were tested for loss of all antibiotic resistances. The majority lost the FRT-flanked resistance gene and the FLP helper plasmid simultaneously. There was also a "FRT" scar leftover. The resulting strain was named ECKh-172.

CS and ACONT are not highly active or highly expressed under anaerobic conditions. To this end, the arcA gene, which encodes for a global regulator of the TCA cycle, was deleted. ArcA works during microaerobic conditions to induce the expression of gene products that allow the activity of central metabolism enzymes that are sensitive to low oxygen levels, aceE, pflB and adhE. It was shown that microaerobically, a deletion in arcA/arcB increases the specific activities of ldh, icd, gltA, mdh, and gdh genes (Salmon et al., *J. Biol. Chem.* 280:15084-15096 (2005); Shalel-Levanon et al., *Biotechnol. Bioeng.* 92(2):147-159 (2005). The upstream and downstream regions of the arcA gene of *E. coli* MG1655 were amplified by PCR using primers ArcA-up-EcoRI (5'-ataataatagaattcgtttgctacctaaattgc-caactaaatcgaaacagg-3') (SEQ ID NO:33) with ArcA-up-KpnI (5'-tattattatggtaccaatatcatgcagcaaacggtgcaacattgccg-3') (SEQ ID NO:34) and ArcA-down-EcoRI (5'-tgatctggaagaattcatcggctttaccaccgtcaaaaaaaacggcg-3') (SEQ ID NO:35) with ArcA-down-PstI (5'-ataaaaccctgcagcg-gaaacgaagttttatccatttttggttacctg-3') (SEQ ID NO:36), respectively. These fragments were subsequently digested with the restriction enzymes EcoRI and KpnI (upstream fragment) and EcoRI and PstI (downstream). They were then ligated into the pRE118-V2 plasmid digested with PstI and KpnI, leading to plasmid pRE118-ΔarcA. The sequence of plasmid pRE118-ΔarcA was verified. pRE118-ΔarcA was introduced into electro-competent cells of *E. coli* strain ECKh-172 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh). After integration and resolution on LB-no salt-sucrose plates as described above, the deletion of the arcA gene in the chromosome of the resulting strain ECKh-401 was verified by sequencing and is shown in FIG. **42**.

The gltA gene of *E. coli* encodes for a citrate synthase. It was previously shown that this gene is inhibited allosterically by NADH, and the amino acids involved in this inhibition have been identified (Pereira et al., *J. Biol. Chem.* 269(1):412-417 (1994); Stokell et al., *J. Biol. Chem.* 278 (37):35435-35443 (2003)). The gltA gene of *E. coli*

MG1655 was amplified by PCR using primers gltA-up (5'-ggaagagaggctggtacccagaagccacagcagga-3') (SEQ ID NO:37) and gltA-PstI (5'-gtaatcactgcgtaagcgccatgccccg-gcgttaattc-3') (SEQ ID NO:38). The amplified fragment was cloned into pRE118-V2 after digestion with KpnI and PstI. The resulting plasmid was called pRE118-gltA. This plasmid was then subjected to site directed mutagenesis (SDM) using primers R163L-f (5'-attgccgcgttcctcctgctgtcga-3') (SEQ ID NO:39) and R163L-r (5'-cgacagcaggaggaacgcg-gcaat-3') (SEQ ID NO:40) to change the residue Arg 163 to a Lys residue. The sequence of the entire fragment was verified by sequencing. A variation of the X red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)) was used to replace the native gltA gene with the R163L mutant allele without leaving a Frt scar. The general recombination procedure is the same as used to make the mdh deletion described above. First, the strain ECKh-172 was made streptomycin resistant by introducing an rpsL null mutation using the λ red homologeous recombination method. Next, a recombination was done to replace the entire wild-type gltA coding region in this strain with a cassette comprised of a kanamycin resistance gene (kanR) and a wild-type copy of the *E. coli* rpsL gene. When introduced into an *E. coli* strain harboring an rpsL null mutation, the cassette causes the cells to change from resistance to the drug streptomycin to streptomycin sensitivity. DNA fragments were then introduced that included each of the mutant versions of the gltA gene along with appropriate homologous ends, and resulting colony growth was tested in the presence of streptomycin. This selected for strains in which the kanR/rpsL cassette had been replaced by the mutant gltA gene. Insertion of the mutant gene in the correct locus was confirmed by PCR and DNA sequencing analyses. The resulting strain was called ECKh-422, and has the genotype ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L. The region encompassing the mutated gltA gene of strain ECKh-422 was verified by sequencing, as shown in FIG. **43**.

Crude extracts of the strains ECKh-401 and the gltAR163L mutant ECKh-422 were then evaluated for citrate synthase activity. Cells were harvested by centrifugation at 4,500 rpm (Beckman-Coulter, Allegera X-15R; Fullerton Calif.) for 10 min. The pellets were resuspended in 0.3 mL BugBuster (Novagen/EMD; San Diego Calif.) reagent with benzonase and lysozyme, and lysis proceeded for 15 minutes at room temperature with gentle shaking Cell-free lysate was obtained by centrifugation at 14,000 rpm (Eppendorf centrifuge 5402; Hamburg Germany) for 30 min at 4° C. Cell protein in the sample was determined using the method of Bradford (Bradford, *Anal. Biochem.* 72:248-254 (1976)).

Citrate synthase activity was determined by following the formation of free coenzyme A (HS-CoA), which is released from the reaction of acetyl-CoA with oxaloacetate. The free thiol group of HS-CoA reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB). The concentration of TNB is then monitored spectrophotometrically by measuring the absorbance at 410 nm (maximum at 412 nm). The assay mixture contained 100 mM Tris/HCl buffer (pH 7.5), 20 mM acetyl-CoA, 10 mM DTNB, and 20 mM oxaloacetate. For the evaluation of NADH inhibition, 0.4 mM NADH was also added to the reaction. The assay was started by adding 5 microliters of the cell extract, and the rate of reaction was measured by following the absorbance change over time. A unit of specific activity is defined as the μmol of product converted per minute per mg protein.

FIG. **44** shows the citrate synthase activity of wild type gltA gene product and the R163L mutant. The assay was performed in the absence or presence of 0.4 mM NADH.

Strains ECKh-401 and ECKh-422 were transformed with plasmids expressing the entire BDO pathway. *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, and *M. bovis* sucA were expressed on the low copy plasmid pZS*13, and *P. gingivalis* Cat2 and *C. acetobutylicum* AdhE2 were expressed on the medium copy plasmid pZE23. Cultures of these strains were grown microaerobically in M9 minimal medium supplemented with 20 g/L glucose and the appropriate antibiotics as described above. The 4HB and BDO concentrations at 48 hours post-induction averaged from duplicate cultures are shown in FIG. **45**. Both are higher in ECKh-422 than in ECKh-401, demonstrating that the enhanced citrate synthase activity due to the gltA mutation results in increased flux to the BDO pathway.

The host strain modifications described in this section were intended to redirect carbon flux through the oxidative TCA cycle, which is consistent with the OptKnock strain design described in WO 2009/023493 and U.S. publication 2009/0047719. To demonstrate that flux was indeed routed through this pathway, ^{13}C flux analysis was performed using the strain ECKh-432, which is a version of ECKh-422 in which the upstream pathway is integrated into the chromosome (as described in Example XVII). To complete the BDO pathway, *P. gingivalis* Cat2 and *C. beijerinckii* Ald were expressed from pZS*13. Four parallel cultures were grown in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4C1, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) containing 4 g/L total glucose of four different labeling ratios (^{13}C, only the first carbon atom in the glucose molecule is labeled with ^{13}C; uniform-^{13}C, all carbon atoms are ^{13}C):

1. 80 mol % unlabeled, 20 mol % uniform-^{13}C
2. 10 mol % unlabeled, 90 mol % uniform-^{13}C
3. 90 mol % ^{13}C, 10 mol % uniform-^{13}C
4. 40 mol % ^{13}C, 60 mol % uniform-^{13}C

Parallel unlabeled cultures were grown in duplicate, from which frequent samples were taken to evaluate growth rate, glucose uptake rate, and product formation rates. In late exponential phase, the labeled cultures were harvested, the protein isolated and hydrolyzed to amino acids, and the label distribution of the amino acids analyzed by gas chromatography-mass spectrometry (GCMS) as described previously (Fischer and Sauer, *Eur. J. Biochem.* 270:880-891 (2003)). In addition, the label distribution of the secreted 4HB and BDO in the broth from the labeled cultures was analyzed by GCMS as described in WO2008115840. This data was collectively used to calculate the intracellular flux distribution using established methods (Suthers et al., *Metab. Eng.* 9:387-405 (2007)). The resulting central metabolic fluxes and associated 95% confidence intervals are shown in FIG. **46**. Values are molar fluxes normalized to a glucose uptake rate of 1 mmol/hr. The result indicates that carbon flux is routed through citrate synthase in the oxidative direction, and that most of the carbon enters the BDO pathway rather than completing the TCA cycle. Furthermore, it confirms there is essentially no flux between malate and oxaloacetate due to the mdh deletion in this strain.

The advantage of using a knockout strain such as strains designed using OptKnock for BDO production (see WO 2009/023493 and U.S. publication 2009/0047719) can be observed by comparing typical fermentation profiles of ECKh-422 with that of the original strain ECKh-138, in which BDO is produced from succinate via the reductive TCA cycle (see FIG. **47**). Fermentations were performed

with 1 L initial culture volume in 2 L Biostat B+ bioreactors (Sartorius; Cedex France) using M9 minimal medium supplemented with 20 g/L glucose. The temperature was controlled at 37° C., and the pH was controlled at 7.0 using 2 M NH_4OH or Na_2CO_3. Cells were grown aerobically to an OD600 of approximately 10, at which time the cultures were induced with 0.2 mM IPTG. One hour following induction, the air flow rate was reduced to 0.02 standard liters per minute for microaerobic conditions. The agitation rate was set at 700 rpm. Concentrated glucose was fed to maintain glucose concentration in the vessel between 0.5 and 10 g/L. Both strains were transformed with plasmids bearing the entire BDO pathway, as in the examples above. In ECKh-138, acetate, pyruvate, and 4HB dominate the fermentation, while with ECKh-422 BDO is the major product.

EXAMPLE XVI

BDO Strains Expression Phosphoenolpyruvate Carboxykinase

This example describes the utilization of phosphoenolpyruvate carboxykinase (PEPCK) to enhance BDO production. The *Haemophilus influenza* PEPCK gene was used for heterologous expression.

Computationally, it was demonstrated that the ATP-generating conversion of oxaloacetate to phosphoenolpyruvate is required to reach the maximum theoretical yield of 1,4-butanediol (see also WO2008/115840, WO 2009/023493, U.S. publication 2009/0047719, U.S. publication 2009/0075351). Lack of PEPCK activity was shown to reduce the maximum theoretical yield of BDO by 12% assuming anaerobic conditions and by 3% assuming an external electron acceptor such as nitrate or oxygen is present.

In organisms such as *E. coli*, PEPCK operates in the gluconeogenic and ATP-consuming direction from oxaloacetate towards phosphoenolpyruvate. It has been hypothesized that kinetic limitations of PEPCK of *E. coli* prevent it from effectively catalyzing the formation of oxaloacetate from PEP. PEP carboxylase (PPC), which does not generate ATP but is required for efficient growth, is naturally utilized by *E. coli* to form oxaloacetate from phosphoenolpyruvate. Therefore, three non native PEPCK enzymes (Table 26) were tested for their ability to complement growth of a PPC mutant strain of *E. coli* in glucose minimal media.

TABLE 26

Sources of phosphoenolpyruvate carboxykinase sequences.

PEPCK Source Strain	Accession Number, GenBank Reference Sequence
Haemophilus influenza	NC_000907.1
Actinobacillus succinogenes	YP_001343536.1
Mannheimia succiniciproducens	YP_089485.1

Growth complementation studies involved plasmid based expression of the candidate genes in Δppc mutant *E. coli* JW3978 obtained from the Keio collection (Baba et al., Molecular Systems Biology 2:2006.0008 (2006)). The genes were cloned behind the PA1lacO-1 promoter in the expression vectors pZA23 (medium copy) and pZE13 (high copy). These plasmids have been described previously (Lutz and Bujard, *Nucleic Acids Res.* 25:1203-1210 (1997)), and their use in expression BDO pathway genes has been described previously in WO2008115840.

Pre-cultures were grown aerobically in M9 minimal media with 4 g/L glucose. All pre-cultures were supplemented with aspartate (2 mM) to provide the Δppc mutants with a source for generating TCA cycle intermediates independent of PEPCK expression. M9 minimal media was also used in the test conditions with 4 g/L glucose, but no aspartate was added and IPTG was added to 0.5 mM. Table 27 shows the results of the growth complementation studies.

TABLE 27

Complementation of Δppc mutants with PEPCK from *H. influenzae*, *A. succinogenes* and *M. succinoproducens* when expressed from vectors pZA23 or pZE13.

PEPCK Source Strain	Vector	Time (h)	OD_{600}
H. influenzae	pZA23BB	40	0.950
Δppc Control	pZA23BB	40	0.038
A. succinogenes	pZA23BB	40	0.055
M. succinoproducens	pZA23BB	40	0.214
A. succinogenes	pZE13BB	40	0.041
M. succinoproducens	pZE13BB	40	0.024
Δppc Control	pZE13BB	40	0.042

Haemophilus influenza PEPCK was found to complement growth in Δppc mutant *E. coli* best among the genes that were tested in the plasmid based screening. This gene was then integrated into the PPC locus of wild-type *E. coli* (MG1655) using the SacB counter selection method with pRE118-V2 discussed above (Gay et al., *J. Bacteriol.* 153: 1424-1431 (1983)). PEPCK was integrated retaining the *E. coli* native PPC promoter, but utilizing the non-native PEPCK terminator. The sequence of this region following replacement of ppc by *H. influenzae* pepck is shown in FIG. **48**. The pepck coding region is underlined.

Techniques for adaptive evolution were applied to improve the growth rate of the *E. coli* mutant (Δppc::H. inf pepCK). M9 minimal media with 4 g/L glucose and 50 mM sodium bicarbonate was used to culture and evolve this strain in an anaerobic environment. The high sodium bicarbonate concentration was used to drive the equilibrium of the PEPCK reaction toward oxaloacetate formation. To maintain exponential growth, the culture was diluted 2-fold whenever an OD600 of 0.5 was achieved. After about 100 generations over 3 weeks of adaptive evolution, anaerobic growth rates improved from about 8 h to that of wild type, about 2 h. Following evolution, individual colonies were isolated, and growth in anaerobic bottles was compared to that of the initial mutant and wild-type strain (see FIG. **49**). M9 medium with 4 g/L glucose and 50 mM sodium bicarbonate was used.

The ppc/pepck gene replacement procedure described above was then repeated, this time using the BDO-producing strains ECKh-432 (ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd) and ECKh-439 as the hosts. These strains contain the TCA cycle enhancements discussed above as well as the upstream pathway integrated in the chromosome. ECKh-439 is a derivative of ECKh-432 that has the ackA gene deleted, which encodes acetate kinase. This deletion was performed using the sacB counterselection method described above.

The Δppc::H. inf pepCK derivative of ECKh-439, called ECKh-453, was run in a fermentation. The downstream BDO pathway was supplied by pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald. This was performed with 1 L initial culture volume in 2 L Biostat B+ bioreactors (Sartorius) using M9 minimal medium supplemented with 20 g/L glucose and 50 mM $NaHCO_3$. The temperature was controlled at 37° C., and the pH was controlled at 7.0 using 2 M NH_4OH or Na_2CO_3. Cells were grown aerobically to an OD600 of approximately 2, at which time the cultures were

induced with 0.2 mM IPTG. One hour following induction, the air flow rate was reduced to 0.01 standard liters per minute for microaerobic conditions. The agitation rate was initially set at 700 rpm. The aeration rate was gradually increased throughout the fermentation as the culture density increased. Concentrated glucose solution was fed to maintain glucose concentration in the vessel between 0.5 and 10 g/L. The product profile is shown in FIG. **50**. The observed phenotype, in which BDO and acetate are produced in approximately a one-to-one molar ratio, is highly similar to that predicted in WO 2009/023493 for design #439 (ADHEr, ASPT, LDH_D, MDH, PFLi). The deletion targeting the ASPT reaction was deemed unnecessary as the natural flux through aspartate ammonia-lyase is low.

A key feature of OptKnock strains is that production of the metabolite of interest is generally coupled to growth, and further, that, production should occur during exponential growth as well as in stationary phase. The growth coupling potential of ECKh-432 and ECKh-453 was evaluated by growth in microaerobic bottles with frequent sampling during the exponential phase. M9 medium containing 4 g/L glucose and either 10 mM $NaHCO_3$ (for ECKh-432) or 50 mM $NaHCO_3$ (for ECKh-453) was used, and 0.2 mM IPTG was included from inoculation. 18G needles were used for microaerobic growth of ECKh-432, while both 18G and 27G needles were tested for ECKh-453. The higher gauge needles result in less aeration. As shown in FIG. **51**, ECKh-432 does not begin producing BDO until 5 g/L glucose has been consumed, corresponding to the onset of stationary phase. ECKh-453 produces BDO more evenly throughout the experiment. In addition, growth coupling improves as the aeration of the culture is reduced.

EXAMPLE XVII

Integration of BDO Pathway Encoding Genes at Specific Integration Sites

This example describes integration of various BDO pathway genes into the fimD locus to provide more efficient expression and stability.

The entire upstream BDO pathway, leading to 4HB, has been integrated into the *E. coli* chromosome at the fimD locus. The succinate branch of the upstream pathway was integrated into the *E. coli* chromosome using the X red homologeous recombination method (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97:6640-6645 (2000)). The recipient *E. coli* strain was ECKh-422 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L). A polycistronic DNA fragment containing a promoter, the sucCD gene, the sucD gene and the 4hbd gene and a terminator sequence was inserted into the AflIII site of the pKD3 plasmid. The following primers were used to amplify the operon together with the chloramphenicol marker from the plasmid. The underlined sequences are homologeous to the target insertion site.

(SEQ ID NO: 41)
5'-GTTTGCACGCTATAGCTGAGGTTGTTGTCTTCCAGCAACGTACCGT

ATACAATAGGCGTATCACGAGGCCCTTTC-3'

(SEQ ID NO: 42)
5'-GCTACAGCATGTCACACGATCTCAACGGTCGGATGACCAATCTGGC

TGGTATGGGAATTAGCCATGGTCC-3'

Following DpnI treatment and DNA electrophoresis, the purified PCR product was used to transform *E. coli* strain harboring plasmid pKD46. The candidate strain was selected on plates containing chloramphenicol. Genomic DNA of the candidate strain was purified. The insertion sequence was amplified and confirmed by DNA sequencing. The chloramphenicol-resistant marker was removed from chromosome by flipase. The nucleotide sequence of the region after insertion and marker removal is shown in FIG. **52**.

The alpha-ketoglutarate branch of the upstream pathway was integrated into the chromosome by homologeous recombination. The plasmid used in this modification was derived from vector pRE118-V2, as referenced in Example XIV, which contains a kanamycin-resistant gene, a gene encoding the levansucrase (sacB) and a R6K conditional replication ori. The integration plasmid also contained a polycistronic sequence with a promoter, the sucA gene, the *C. kluyveri* 4hbd gene, and a terminator being inserted between two 1.5-kb DNA fragments that are homologeous to the flanking regions of the target insertion site. The resulting plasmid was used to transform *E. coli* strain. The integration candidate was selected on plates containing kanamycin. The correct integration site was verified by PCR. To resolve the antibiotic marker from the chromosome, the cells were selected for growth on medium containing sucrose. The final strain was verified by PCR and DNA sequencing. The nucleotide sequence of the chromosomal region after insertion and marker removal is shown in FIG. **53**.

The resulting upstream pathway integration strain ECKh-432 was transformed with a plasmid harboring the downstream pathway genes. The construct was able to produce BDO from glucose in minimal medium (see FIG. **54**).

EXAMPLE XVIII

Use of a Non-Phosphotransferase Sucrose Uptake System to Reduce Pyruvate Byproduct Formation

This example describes the utilization of a non-phosphotransferase (PTS) sucrose uptake system to reduce pyruvate as a byproduct in the conversion of sucrose to BDO.

Strains engineered for the utilization of sucrose via a phosphotransferase (PTS) system produce significant amounts of pyruvate as a byproduct. Therefore, the use of a non-PTS sucrose system can be used to decrease pyruvate formation because the import of sucrose would not be accompanied by the conversion of phosphoenolpyruvate (PEP) to pyruvate. This will increase the PEP pool and the flux to oxaloacetate through PPC or PEPCK.

Insertion of a non-PTS sucrose operon into the rrnC region was performed. To generate a PCR product containing the non-PTS sucrose genes flanked by regions of homology to the rrnC region, two oligos were used to PCR amplify the csc genes from Mach1™ (Invitrogen, Carlsbad, Calif.). This strain is a descendent of W strain which is an *E. coli* strain known to be able to catabolize sucrose (Orencio-Trejo et al., *Biotechnology Biofuels* 1:8 (2008)). The sequence was derived from *E. coli* W strain KO11 (accession AY314757) (Shukla et al., *Biotechnol. Lett.* 26:689-693 (2004)) and includes genes encoding a sucrose permease (cscB), D-fructokinase (cscK), sucrose hydrolase (cscA), and a LacI-related sucrose-specific repressor (cscR). The first 53 amino acids of cscR was effectively removed by the placement of the AS primer. The sequences of the oligos were: rrnC 23S del S-CSC 5'-TGT GAG TGA AAG TCA CCT GCC TTA ATA TCT CAA AAC TCA TCT TCG GGT GA CGAAATATGGCGTGACTCGATAC-3' (SEQ ID NO:43)

and rrnC 23S del AS-CSC 5'-TCT GTA TCA GGC TGA AAA TCT TCT CTC ATC CGC CAA AAC AGC TTC GGC GTTAAGATGCGCGCTCAAGGAC-3' (SEQ ID NO:44). Underlined regions indicate homology to the csc operon, and bold sequence refers to sequence homology upstream and downstream of the rrnC region. The sequence of the entire PCR product is shown in FIG. **55**.

After purification, the PCR product was electroporated into MG1655 electrocompetent cells which had been transformed with pRedET (tet) and prepared according to manufacturer's instructions (genebridges.com/gb/pdf/K001%20Q%20E%20BAC%20Modification%20Kit-version2.6-2007-screen.pdf). The PCR product was designed so that it integrated into genome into the rrnC region of the chromosome. It effectively deleted 191 nucleotides upstream of rrlC (23S rRNA), all of the rrlC rRNA gene and 3 nucleotides downstream of rrlC and replaced it with the sucrose operon, as shown in FIG. **56**.

Transformants were grown on M9 minimal salts medium with 0.4% sucrose and individual colonies tested for presence of the sucrose operon by diagnostic PCR. The entire rrnC::crcAKB region was transferred into the BDO host strain ECKh-432 by P1 transduction (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001), resulting in ECKh-463 (ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd rrnC::cscAKB). Recombinants were selected by growth on sucrose and verified by diagnostic PCR.

ECKh-463 was transformed with pZS*13 containing *P. gingivalis* Cat2 and *C. beijerinckii* Ald to provide a complete BDO pathway. Cells were cultured in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4C1, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 10 g/L sucrose. 0.2 mM IPTG was present in the culture from the start. Anaerobic conditions were maintained using a bottle with 23G needle. As a control, ECKh-432 containing the same plasmid was cultured on the same medium, except with 10 g/L glucose instead of sucrose. FIG. **57** shows average product concentration, normalized to culture OD600, after 48 hours of growth. The data is for 6 replicate cultures of each strain. This demonstrates that BDO production from ECKh-463 on sucrose is similar to that of the parent strain on sucrose.

EXAMPLE XIX

Summary of BDO Producing Strains

This example describes various BDO producing strains. Table 28 summarizes various BDO producing strains disclosed above in Examples XII-XVIII.

TABLE 28

Summary of various BDO production strains.

Strain #	Host Strain #	Host chromosome	Host Description	Plasmid-based
1		ΔldhA	Single deletion derivative of *E. coli* MG1655	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
2	AB3	ΔadhE ΔldhA ΔpflB	Succinate producing strain; derivative of *E. coli* MG1655	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
3	ECKh-138	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322	Improvement of lpdA to increase pyruvate dehydrogenase flux	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
4	ECKh-138	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322		*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. acetobutylicum* AdhE2
5	ECKh-401	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA	Deletions in mdh and arcA to direct flux through oxidative TCA cycle	*E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
6	ECKh-401	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
7	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA gltAR163L	Mutation in citrate synthase to improve anaerobic activity	*E. coli sucCD, P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
8	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA gltAR163L		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2
9	ECKh-422	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 ΔmdhΔarcA gltAR163L		*M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. beijerinckii* Ald

TABLE 28-continued

Summary of various BDO production strains.

Strain #	Host Strain #	Host chromosome	Host Description	Plasmid-based
10	ECKh-426	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis sucD, P. gingivalis* 4hbd	Succinate branch of upstream pathway integrated into ECKh-422	*P. gingivalis* Cat2, *C. beijerinckii* Ald
11	ECKh-432	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis sucD, P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Succinate and alpha-ketoglutarate upstream pathway branches integrated into ECKh-422	*P. gingivalis Cat2, C. beijerinckii* Ald
12	ECKh-432	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, P. gingivalis sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd		*C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald
13	ECKh-439	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Acetate kinase deletion of ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
14	ECKh-453	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L ΔackA Δppc::H.i.ppck fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Acetate kinase deletion and PPC/PEPCK replacement of ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
15	ECKh-456	ΔadhE ΔldhA ΔpflB ΔlpdA::fnr-pflB6-K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Replacement of lpdA promoter with anaerobic promoter in ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
16	ECKh-455	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 ΔpdhR:: fnr-pflB6 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, P. gingivalis 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd	Replacement of pdhR and aceEF promoter with anaerobic promoter in ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
17	ECKh-459	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd fimD:: *C. acetobutylicum* buk1, *C. acetobutylicum* ptb	Integration of BK/PTB into ECKh-432	*C. beijerinckii* Ald
18	ECKh-459	ΔadhE ΔldhA ΔpflB ΔlpdA:: K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd fimD:: *C. acetobutylicum* buk1, *C. acetobutylicum* ptb		*C. beijerinckii* Ald, *G. thermoglucosidasius* adh1
19	ECKh-463	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: E. coli sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd rrnC::cscAKB	Non-PTS sucrose genes inserted into ECKh-432	*P. gingivalis* Cat2, *C. beijerinckii* Ald
20	ECKh-463	ΔadhE ΔldhA ΔpflB ΔlpdA::K.p.lpdA322 Δmdh ΔarcA gltAR163L fimD:: *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd fimD:: *M. bovis* sucA, *C. kluyveri* 4hbd rrnC::cscAKB		*C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald

The strains summarized in Table 28 are as follows. Strain 1: Single deletion derivative of *E. coli* MG1655, with deletion of endogenous ldhA; plasmid expression of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2. Strain 2: Host strain AB3, a succinate producing strain, derivative of *E. coli* MG1655, with deletions of endogenous adhE ldhA pflB; plasmid expression of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2.

Strain 3: Host strain ECKh-138, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus; plasmid expression of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2; strain provides improvement of lpdA to increase pyruvate dehydrogenase flux. Strain 4: Host strain ECKh-138, deletion of endogenous adhE, ldhA, pflB, and lpdA, chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation; plasmid expression *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *C. acetobutylicum* buk1, *C. acetobutylicum* ptb, *C. acetobutylicum* AdhE2.

Strain 5: Host strain ECKh-401, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA; plasmid expression of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2; strain has deletions in mdh and arcA to direct flux through oxidative TCA cycle. Strain 6: host strain ECKh-401, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA; plasmid expression of *M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2.

Strain 7: Host strain ECKh-422, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant; plasmid expression of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2; strain has mutation in citrate synthase to improve anaerobic activity. Strain 8: strain ECKh-422, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant; plasmid expression of *M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. acetobutylicum* AdhE2. Strain 9: host strain ECKh-422, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant; plasmid expression of *M. bovis* sucA, *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, *P. gingivalis* Cat2, *C. beijerinckii* Ald.

Strain 10: host strain ECKh-426, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has succinate branch of upstream pathway integrated into strain ECKh-422 at the fimD locus. Strain 11: host strain ECKh-432, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has succinate and alpha-ketoglutarate upstream pathway branches integrated into ECKh-422. Strain 12: host strain ECKh-432, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd; plasmid expression of *C. acetobutylicum buk*1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald.

Strain 13: host strain ECKh-439, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, deletion of endogenous ackA, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has acetate kinase deletion in strain ECKh-432. Strain 14: host strain ECKh-453, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, deletion of endogenous ackA, deletion of endogenous ppc and insertion of *Haemophilus influenza* ppck at the ppc locus, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has acetate kinase deletion and PPC/PEPCK replacement in strain ECKh-432.

Strain 15: host strain ECKh-456, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd, replacement of lpdA promoter with fnr binding site, pflB-p6 promoter and RBS of pflB; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has replacement of lpdA promoter with anaerobic promoter in strain ECKh-432. Strain 16: host strain ECKh-455, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus,

deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbdI, replacement of pdhR and aceEF promoter with fnr binding site, pflB-p6 promoter and RBS of pflB; plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has replacement of pdhR and aceEF promoter with anaerobic promoter in ECKh-432.

Strain 17: host strain ECKh-459, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd, chromosomal insertion at the fimD locus of *C. acetobutylicum buk*1, *C. acetobutylicum* ptb; plasmid expression of *C. beijerinckii* Ald; strain has integration of BK/PTB into strain ECKh-432. Strain 18: host strain ECKh-459, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd, chromosomal insertion at the fimD locus of *C. acetobutylicum* buk1, *C. acetobutylicum* ptb; plasmid expression of *C. beijerinckii* Ald, *G. thermoglucosidasius* adh1.

Strain 19: host strain ECKh-463, deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd, insertion at the rrnC locus of non-PTS sucrose operon genes sucrose permease (cscB), D-fructokinase (cscK), sucrose hydrolase (cscA), and a LacI-related sucrose-specific repressor (cscR); plasmid expression of *P. gingivalis* Cat2, *C. beijerinckii* Ald; strain has non-PTS sucrose genes inserted into strain ECKh-432. Strain 20: host strain ECKh-463 deletion of endogenous adhE, ldhA, pflB, deletion of endogenous lpdA and chromosomal insertion of *Klebsiella pneumoniae* lpdA with a Glu354Lys mutation at the lpdA locus, deletion of endogenous mdh and arcA, chromosomal replacement of gltA with gltA Arg163Leu mutant, chromosomal insertion at the fimD locus of *E. coli* sucCD, *P. gingivalis* sucD, *P. gingivalis* 4hbd, chromosomal insertion at the fimD locus of *M. bovis* sucA, *C. kluyveri* 4hbd, insertion at the rrnC locus of non-PTS sucrose operon; plasmid expression of *C. acetobutylicum buk*1, *C. acetobutylicum* ptb, *C. beijerinckii* Ald.

In addition to the BDO producing strains disclosed herein, including those disclosed in Table 28, it is understood that additional modifications can be incorporated that further increase production of BDO and/or decrease undesirable byproducts. For example, a BDO producing strain, or a strain of Table 28, can incorporate additional knockouts to further increase the production of BDO or decrease an undesirable byproduct. Exemplary knockouts have been described previously (see U.S. publication 2009/0047719). Such knockout strains include, but are not limited to, ADHEr, NADH6; ADHEr, PPCK; ADHEr, SUCD4; ADHEr, ATPS4r; ADHEr, FUM; ADHEr, MDH; ADHEr, PFLi, PPCK; ADHEr, PFLi, SUCD4; ADHEr, ACKr, NADH6; ADHEr, NADH6, PFLi; ADHEr, ASPT, MDH; ADHEr, NADH6, PPCK; ADHEr, PPCK, THD2; ADHEr, ATPS4r, PPCK; ADHEr, MDH, THD2; ADHEr, FUM, PFLi; ADHEr, PPCK, SUCD4; ADHEr, GLCpts, PPCK; ADHEr, GLUDy, MDH; ADHEr, GLUDy, PPCK; ADHEr, FUM, PPCK; ADHEr, MDH, PPCK; ADHEr, FUM, GLUDy; ADHEr, FUM, HEX1; ADHEr, HEX1, PFLi; ADHEr, HEX1, THD2; ADHEr, FRD2, LDH_D, MDH; ADHEr, FRD2, LDH_D, ME2; ADHEr, MDH, PGL, THD2; ADHEr, G6PDHy, MDH, THD2; ADHEr, PFLi, PPCK, THD2; ADHEr, ACKr, AKGD, ATPS4r; ADHEr, GLCpts, PFLi, PPCK; ADHEr, ACKr, ATPS4r, SUCOAS; ADHEr, GLUDy, PFLi, PPCK; ADHEr, ME2, PFLi, SUCD4; ADHEr, GLUDy, PFLi, SUCD4; ADHEr, ATPS4r, LDH_D, SUCD4; ADHEr, FUM, HEX1, PFLi; ADHEr, MDH, NADH6, THD2; ADHEr, ATPS4r, MDH, NADH6; ADHEr, ATPS4r, FUM, NADH6; ADHEr, ASPT, MDH, NADH6; ADHEr, ASPT, MDH, THD2; ADHEr, ATPS4r, GLCpts, SUCD4; ADHEr, ATPS4r, GLUDy, MDH; ADHEr, ATPS4r, MDH, PPCK; ADHEr, ATPS4r, FUM, PPCK; ADHEr, ASPT, GLCpts, MDH; ADHEr, ASPT, GLUDy, MDH; ADHEr, ME2, SUCD4, THD2; ADHEr, FUM, PPCK, THD2; ADHEr, MDH, PPCK, THD2; ADHEr, GLUDy, MDH, THD2; ADHEr, HEX1, PFLi, THD2; ADHEr, ATPS4r, G6PDHy, MDH; ADHEr, ATPS4r, MDH, PGL; ADHEr, ACKr, FRD2, LDH_D; ADHEr, ACKr, LDH_D, SUCD4; ADHEr, ATPS4r, FUM, GLUDy; ADHEr, ATPS4r, FUM, HEX1; ADHEr, ATPS4r, MDH, THD2; ADHEr, ATPS4r, FRD2, LDH_D; ADHEr, ATPS4r, MDH, PGDH; ADHEr, GLCpts, PPCK, THD2; ADHEr, GLUDy, PPCK, THD2; ADHEr, FUM, HEX1, THD2; ADHEr, ATPS4r, ME2, THD2; ADHEr, FUM, ME2, THD2; ADHEr, GLCpts, GLUDy, PPCK; ADHEr, ME2, PGL, THD2; ADHEr, G6PDHy, ME2, THD2; ADHEr, ATPS4r, FRD2, LDH_D, ME2; ADHEr, ATPS4r, FRD2, LDH_D, MDH; ADHEr, ASPT, LDH_D, MDH, PFLi; ADHEr, ATPS4r, GLCpts, NADH6, PFLi; ADHEr, ATPS4r, MDH, NADH6, PGL; ADHEr, ATPS4r, G6PDHy, MDH, NADH6; ADHEr, ACKr, FUM, GLUDy, LDH_D; ADHEr, ACKr, GLUDy, LDH_D, SUCD4; ADHEr, ATPS4r, G6PDHy, MDH, THD2; ADHEr, ATPS4r, MDH, PGL, THD2; ADHEr, ASPT, G6PDHy, MDH, PYK; ADHEr, ASPT, MDH, PGL, PYK; ADHEr, ASPT, LDH_D, MDH, SUCOAS; ADHEr, ASPT, FUM, LDH_D, MDH; ADHEr, ASPT, LDH_D, MALS, MDH; ADHEr, ASPT, ICL, LDH_D, MDH; ADHEr, FRD2, GLUDy, LDH_D, PPCK; ADHEr, FRD2, LDH_D, PPCK, THD2; ADHEr, ACKr, ATPS4r, LDH_D, SUCD4; ADHEr, ACKr, ACS, PPC, PPCK; ADHEr, GLUDy, LDH_D, PPC, PPCK; ADHEr, LDH_D, PPC, PPCK, THD2; ADHEr, ASPT, ATPS4r, GLCpts, MDH; ADHEr, G6PDHy, MDH, NADH6, THD2; ADHEr, MDH, NADH6, PGL, THD2; ADHEr, ATPS4r, G6PDHy, GLCpts, MDH; ADHEr, ATPS4r, GLCpts, MDH, PGL; ADHEr, ACKr, LDH_D, MDH, SUCD4.

Table 29 shows the reactions of corresponding genes to be knocked out of a host organism such as *E. coli*. The corresponding metabolite corresponding to abbreviations in Table 29 are shown in Table 30.

TABLE 29

Corresponding genes to be knocked out to prevent a particular reaction from occurring in *E. coli*.

Reaction Abbreviation	Reaction Stoichiometry*	Genes Encoding the Enzyme(s) Catalyzing Each Reaction&
ACKr	[c]: ac + atp <==> actp + adp	(b3115 or b2296 or b1849)
ACS	[c]: ac + atp + coa --> accoa + amp + ppi	b4069
ACt6	ac[p] + h[p] <==> ac[c] + h[c]	Non-gene associated
ADHEr	[c]: etoh + nad <==> acald + h + nadh	(b0356 or b1478 or b1241)
	[c]: acald + coa + nad <==> accoa + h + nadh	(b1241 or b0351)
AKGD	[c]: akg + coa + nad --> co2 + nadh + succoa	(b0116 and b0726 and b0727)
ASNS2	[c]: asp-L + atp + nh4 --> amp + asn-L + h + ppi	b3744
ASPT	[c]: asp-L --> fum + nh4	b4139
ATPS4r	adp[c] + (4) h[p] + pi[c] <==> atp[c] + (3) h[c] + h2o[c]	(((b3736 and b3737 and b3738) and (b3731 and b3732 and b3733 and b3734 and b3735)) or ((b3736 and b3737 and b3738) and (b3731 and b3732 and b3733 and b3734 and b3735) and b3739))
CBMK2	[c]: atp + co2 + nh4 <==> adp + cbp + (2) h	(b0521 or b0323 or b2874)
EDA	[c]: 2ddg6p --> g3p + pyr	b1850
ENO	[c]: 2pg <==> h2o + pep	b2779
FBA	[c]: fdp <==> dhap + g3p	(b2097 or b2925 or b1773)
FBP	[c]: fdp + h2o --> f6p + pi	(b4232 or b3925)
FDH2	for[p] + (2) h[c] + q8[c] --> co2[c] + h[p] + q8h2[c] for[p] + (2) h[c] + mqn8[c] --> co2[c] + h[p] + mql8[c]	((b3892 and b3893 and b3894) or (b1474 and b1475 and b1476))
FRD2	[c]: fum + mql8 --> mqn8 + succ [c]: 2dmmql8 + fum --> 2dmmq8 + succ	(b4151 and b4152 and b4153 and b4154)
FTHFD	[c]: 10fthf + h2o --> for + h + thf	b1232
FUM	[c]: fum + h2o <==> mal-L	(b1612 or b4122 or b1611)
G5SD	[c]: glu5p + h + nadph --> glu5sa + nadp + pi	b0243
G6PDHy	[c]: g6p + nadp <==> 6pgl + h + nadph	b1852
GLCpts	glc-D[p] + pep[c] --> g6p[c] + pyr[c]	((b2417 and b1101 and b2415 and b2416) or (b1817 and b1818 and b1819 and b2415 and b2416) or (b2417 and b1621 and b2415 and b2416))
GLU5K	[c]: atp + glu-L --> adp + glu5p	b0242
GLUDy	[c]: glu-L + h2o + nadp <==> akg + h + nadph + nh4	b1761
GLYCL	[c]: gly + nad + thf --> co2 + mlthf + nadh + nh4	(b2904 and b2903 and b2905 and b0116)
HEX1	[c]: atp + glc-D --> adp + g6p + h	b2388
ICL	[c]: icit --> glx + succ	b4015
LDH_D	[c]: lac-D + nad <==> h + nadh + pyr	(b2133 or b1380)
MALS	[c]: accoa + glx + h2o --> coa + h + mal-L	(b4014 or b2976)
MDH	[c]: mal-L + nad <==> h + nadh + oaa	b3236
ME2	[c]: mal-L + nadp --> co2 + nadph + pyr	b2463
MTHFC	[c]: h2o + methf <==> 10fthf + h	b0529
NADH12	[c]: h + mqn8 + nadh --> mql8 + nad [c]: h + nadh + q8 --> nad + q8h2 [c]: 2dmmq8 + h + nadh --> 2dmmql8 + nad	b1109
NADH6	(4) h[c] + nadh[c] + q8[c] --> (3) h[p] + nad[c] + q8h2[c] (4) h[c] + mqn8[c] + nadh[c] --> (3) h[p] + mql8[c] + nad[c] 2dmmq8[c] + (4) h[c] + nadh[c] --> 2dmmql8[c] + (3) h[p] + nad[c]	(b2276 and b2277 and b2278 and b2279 and b2280 and b2281 and b2282 and b2283 and b2284 and b2285 and b2286 and b2287 and b2288)
PFK	[c]: atp + f6p --> adp + fdp + h	(b3916 or b1723)
PFLi	[c]: coa + pyr --> accoa + for	(((b0902 and b0903) and b2579) or (b0902 and b0903) or (b0902 and b3114) or (b3951 and b3952))
PGDH	[c]: 6pgc + nadp --> co2 + nadph + ru5p-D	b2029
PGI	[c]: g6p <==> f6p	b4025
PGL	[c]: 6pgl + h2o --> 6pgc + h	b0767
PGM	[c]: 2pg <==> 3pg	(b3612 or b4395 or b0755)
PPC	[c]: co2 + h2o + pep --> h + oaa + pi	b3956
PPCK	[c]: atp + oaa --> adp + co2 + pep	b3403
PRO1z	[c]: fad + pro-L --> 1pyr5c + fadh2 + h	b1014
PYK	[c]: adp + h + pep --> atp + pyr	b1854 or b1676)
PYRt2	h[p] + pyr[p] <==> h[c] + pyr[c]	Non-gene associated
RPE	[c]: ru5p-D <==> xu5p-D	(b4301 or b3386)
SO4t2	so4[e] <==> so4[p]	(b0241 or b0929 or b1377 or b2215)

TABLE 29-continued

Corresponding genes to be knocked out to prevent a particular reaction from occurring in *E. coli*.

Reaction Abbreviation	Reaction Stoichiometry*	Genes Encoding the Enzyme(s) Catalyzing Each Reaction&
SUCD4	[c]: q8 + succ --> fum + q8h2	(b0721 and b0722 and b0723 and b0724)
SUCOAS	[c]: atp + coa + succ <==> adp + pi + succoa	(b0728 and b0729)
SULabc	atp[c] + h2o[c] + so4[p] --> adp[c] + h[c] + pi[c] + so4[c]	((b2422 and b2425 and b2424 and b2423) or (b0763 and b0764 and b0765) or (b2422 and b2424 and b2423 and b3917))
TAL	[c]: g3p + s7p <==> e4p + f6p	(b2464 or b0008)
THD2	(2) h[p] + nadh[c] + nadp[c] --> (2) h[c] + nad[c] + nadph[c]	(b1602 and b1603)
THD5	[c]: nad + nadph --> nadh + nadp	(b3962 or (b1602 and b1603))
TPI	[c]: dhap <==> g3p	b3919

TABLE 30

Metabolite names corresponding to abbreviations used in Table 29.

Metabolite Abbreviation	Metabolite Name
10fthf	10-Formyltetrahydrofolate
1pyr5c	1-Pyrroline-5-carboxylate
2ddg6p	2-Dehydro-3-deoxy-D-gluconate 6-phosphate
2dmmq8	2-Demethylmenaquinone 8
2dmmql8	2-Demethylmenaquinol 8
2pg	D-Glycerate 2-phosphate
3pg	3-Phospho-D-glycerate
6pgc	6-Phospho-D-gluconate
6pgl	6-phospho-D-glucono-1,5-lactone
ac	Acetate
acald	Acetaldehyde
accoa	Acetyl-CoA
actp	Acetyl phosphate
adp	ADP
akg	2-Oxoglutarate
amp	AMP
asn-L	L-Asparagine
asp-L	L-Aspartate
atp	ATP
cbp	Carbamoyl phosphate
co2	CO2
coa	Coenzyme A
dhap	Dihydroxyacetone phosphate
e4p	D-Erythrose 4-phosphate
etoh	Ethanol
f6p	D-Fructose 6-phosphate
fad	Flavin adenine dinucleotide oxidized
fadh2	Flavin adenine dinucleotide reduced
fdp	D-Fructose 1,6-bisphosphate
for	Formate
fum	Fumarate
g3p	Glyceraldehyde 3-phosphate
g6p	D-Glucose 6-phosphate
glc-D	D-Glucose
glu5p	L-Glutamate 5-phosphate
glu5sa	L-Glutamate 5-semialdehyde
glu-L	L-Glutamate
glx	Glyoxylate
gly	Glycine
h	H+
h2o	H2O
icit	Isocitrate
lac-D	D-Lactate
mal-L	L-Malate
methf	5,10-Methenyltetrahydrofolate
mlthf	5,10-Methylenetetrahydrofolate
mql8	Menaquinol 8
mqn8	Menaquinone 8
nad	Nicotinamide adenine dinucleotide
nadh	Nicotinamide adenine dinucleotide—reduced
nadp	Nicotinamide adenine dinucleotide phosphate
nadph	Nicotinamide adenine dinucleotide phosphate—reduced
nh4	Ammonium
oaa	Oxaloacetate
pep	Phosphoenolpyruvate
pi	Phosphate
ppi	Diphosphate
pro-L	L-Proline
pyr	Pyruvate
q8	Ubiquinone-8
q8h2	Ubiquinol-8
ru5p-D	D-Ribulose 5-phosphate
s7p	Sedoheptulose 7-phosphate
so4	Sulfate
succ	Succinate
succoa	Succinyl-CoA
thf	5,6,7,8-Tetrahydrofolate
xu5p-D	D-Xylulose 5-phosphate

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

SEQUENCE LISTING

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<400> SEQUENCE: 11

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<400> SEQUENCE: 30

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<210> SEQ ID NO 31
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gagctgcttc 70

<210> SEQ ID NO 32
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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 32

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tcctccttag 70

<210> SEQ ID NO 33
<211> LENGTH: 51
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 33

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<210> SEQ ID NO 34
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 34

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<210> SEQ ID NO 35
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 35

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<400> SEQUENCE: 36

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<210> SEQ ID NO 37
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tcacgaggcc ctttc 75

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gccatggtcc 70

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 43

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cgtgactcga tac 73

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<400> SEQUENCE: 44

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gctcaaggac 70

<210> SEQ ID NO 45
<211> LENGTH: 2036
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<213> ORGANISM: Escherichia coli

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ggtccgtggg tagtgaaatg tcaggttcac gctggtggcc gcggtaaagc gggcggtgtg 180

aaagttgtaa acagcaaaga agacatccgt gcttttgcag aaaactggct gggcaagcgt 240

ctggtaacgt atcaaacaga tgccaatggc caaccggtta accagattct ggttgaagca 300

gcgaccgata tcgctaaaga gctgtatctc ggtgccgttg ttgaccgtag ttcccgtcgt 360

gtggtcttta tggcctccac cgaaggcggc gtggaaatcg aaaaagtggc ggaagaaact 420

ccgcacctga tccataaagt tgcgcttgat ccgctgactg gcccgatgcc gtatcaggga 480

cgcgagctgg cgttcaaact gggtctggaa ggtaaactgg ttcagcagtt caccaaaatc 540

ttcatgggcc tggcgaccat tttcctggag cgcgacctgg cgttgatcga aatcaacccg 600

ctggtcatca ccaaacaggg cgatctgatt tgcctcgacg gcaaactggg cgctgacggc 660

aacgcactgt tccgccagcc tgatctgcgc gaaatgcgtg accagtcgca ggaagatccg 720

cgtgaagcac aggctgcaca gtgggaactg aactacgttg cgctggacgg taacatcggt 780

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agtcgacctg tgtcggtatc ggcggtgacc cgatcccggg ctctaacttt atcgacattc 1740

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gttacatcgc tggtgtgact gcgccgaaag gcaaacgtat gggccacgcg ggtgccatca 1920

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<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 46

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Leu Pro Ala Pro Val Gly Tyr Ala Cys Thr Thr Pro Arg Glu Ala Glu
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Glu Ala Ala Ser Lys Ile Gly Ala Gly Pro Trp Val Val Lys Cys Gln
35 40 45

Val His Ala Gly Gly Arg Gly Lys Ala Gly Gly Val Lys Val Val Asn
50 55 60

Ser Lys Glu Asp Ile Arg Ala Phe Ala Glu Asn Trp Leu Gly Lys Arg
65 70 75 80

Leu Val Thr Tyr Gln Thr Asp Ala Asn Gly Gln Pro Val Asn Gln Ile
85 90 95

Leu Val Glu Ala Ala Thr Asp Ile Ala Lys Glu Leu Tyr Leu Gly Ala
100 105 110

Val Val Asp Arg Ser Ser Arg Arg Val Val Phe Met Ala Ser Thr Glu
115 120 125

Gly Gly Val Glu Ile Glu Lys Val Ala Glu Glu Thr Pro His Leu Ile
130 135 140

His Lys Val Ala Leu Asp Pro Leu Thr Gly Pro Met Pro Tyr Gln Gly
145 150 155 160

Arg Glu Leu Ala Phe Lys Leu Gly Leu Glu Gly Lys Leu Val Gln Gln
165 170 175

Phe Thr Lys Ile Phe Met Gly Leu Ala Thr Ile Phe Leu Glu Arg Asp
180 185 190

Leu Ala Leu Ile Glu Ile Asn Pro Leu Val Ile Thr Lys Gln Gly Asp
195 200 205

Leu Ile Cys Leu Asp Gly Lys Leu Gly Ala Asp Gly Asn Ala Leu Phe
210 215 220

Arg Gln Pro Asp Leu Arg Glu Met Arg Asp Gln Ser Gln Glu Asp Pro
225 230 235 240

Arg Glu Ala Gln Ala Ala Gln Trp Glu Leu Asn Tyr Val Ala Leu Asp
245 250 255

Gly Asn Ile Gly Cys Met Val Asn Gly Ala Gly Leu Ala Met Gly Thr
260 265 270

-continued

```
Met Asp Ile Val Lys Leu His Gly Gly Glu Pro Ala Asn Phe Leu Asp
        275                 280                 285

Val Gly Gly Gly Ala Thr Lys Glu Arg Val Thr Glu Ala Phe Lys Ile
    290                 295                 300

Ile Leu Ser Asp Asp Lys Val Lys Ala Val Leu Val Asn Ile Phe Gly
305                 310                 315                 320

Gly Ile Val Arg Cys Asp Leu Ile Ala Asp Gly Ile Ile Gly Ala Val
                325                 330                 335

Ala Glu Val Gly Val Asn Val Pro Val Val Val Arg Leu Glu Gly Asn
            340                 345                 350

Asn Ala Glu Leu Gly Ala Lys Lys Leu Ala Asp Ser Gly Leu Asn Ile
        355                 360                 365

Ile Ala Ala Lys Gly Leu Thr Asp Ala Ala Gln Gln Val Val Ala Ala
    370                 375                 380

Val Glu Gly Lys
385


<210> SEQ ID NO 47
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 47

Met Ser Ile Leu Ile Asp Lys Asn Thr Lys Val Ile Cys Gln Gly Phe
1               5                   10                  15

Thr Gly Ser Gln Gly Thr Phe His Ser Glu Gln Ala Ile Ala Tyr Gly
            20                  25                  30

Thr Lys Met Val Gly Gly Val Thr Pro Gly Lys Gly Gly Thr Thr His
        35                  40                  45

Leu Gly Leu Pro Val Phe Asn Thr Val Arg Glu Ala Val Ala Ala Thr
    50                  55                  60

Gly Ala Thr Ala Ser Val Ile Tyr Val Pro Ala Pro Phe Cys Lys Asp
65                  70                  75                  80

Ser Ile Leu Glu Ala Ile Asp Ala Gly Ile Lys Leu Ile Ile Thr Ile
                85                  90                  95

Thr Glu Gly Ile Pro Thr Leu Asp Met Leu Thr Val Lys Val Lys Leu
            100                 105                 110

Asp Glu Ala Gly Val Arg Met Ile Gly Pro Asn Cys Pro Gly Val Ile
        115                 120                 125

Thr Pro Gly Glu Cys Lys Ile Gly Ile Gln Pro Gly His Ile His Lys
    130                 135                 140

Pro Gly Lys Val Gly Ile Val Ser Arg Ser Gly Thr Leu Thr Tyr Glu
145                 150                 155                 160

Ala Val Lys Gln Thr Thr Asp Tyr Gly Phe Gly Gln Ser Thr Cys Val
                165                 170                 175

Gly Ile Gly Gly Asp Pro Ile Pro Gly Ser Asn Phe Ile Asp Ile Leu
            180                 185                 190

Glu Met Phe Glu Lys Asp Pro Gln Thr Glu Ala Ile Val Met Ile Gly
        195                 200                 205

Glu Ile Gly Gly Ser Ala Glu Glu Glu Ala Ala Ala Tyr Ile Lys Glu
    210                 215                 220

His Val Thr Lys Pro Val Val Gly Tyr Ile Ala Gly Val Thr Ala Pro
225                 230                 235                 240

Lys Gly Lys Arg Met Gly His Ala Gly Ala Ile Ile Ala Gly Gly Lys
```

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```
               245                 250                 255

Gly Thr Ala Asp Glu Lys Phe Ala Ala Leu Glu Ala Ala Gly Val Lys
            260                 265                 270

Thr Val Arg Ser Leu Ala Asp Ile Gly Glu Ala Leu Lys Thr Val Leu
        275                 280                 285

Lys
```

<210> SEQ ID NO 48
<211> LENGTH: 3696
<212> TYPE: DNA
<213> ORGANISM: Mycobacterium bovis

<400> SEQUENCE: 48

```
atggccaaca taagttcacc attcgggcaa aacgaatggc tggttgaaga gatgtaccgc     60

aagttccgcg acgacccctc ctcggtcgat cccagctggc acgagttcct ggttgactac    120

agccccgaac ccacctccca accagctgcc gaaccaaccc gggttacctc gccactcgtt    180

gccgagcggg ccgctgcggc cgccccgcag gcacccccca agccggccga caccgcggcc    240

gcgggcaacg gcgtggtcgc cgcactggcc gccaaaactg ccgttccccc gccagccgaa    300

ggtgacgagg tagcggtgct gcgcggcgcc gccgcggccg tcgtcaagaa catgtccgcg    360

tcgttggagg tgccgacggc gaccagcgtc cgggcggtcc cggccaagct actgatcgac    420

aaccggatcg tcatcaacaa ccagttgaag cggacccgcg gcggcaagat ctcgttcacg    480

catttgctgg gctacgccct ggtgcaggcg gtgaagaaat tcccgaacat gaaccggcac    540

tacaccgaag tcgacggcaa gcccaccgcg gtcacgccgg cgcacaccaa tctcggcctg    600

gcgatcgacc tgcaaggcaa ggacgggaag cgttccctgg tggtggccgg catcaagcgg    660

tgcgagacca tgcgattcgc gcagttcgtc acggcctacg aagacatcgt acgccgggcc    720

cgcgacggca agctgaccac tgaagacttt gccggcgtga cgatttcgct gaccaatccc    780

ggaaccatcg gcaccgtgca ttcggtgccg cggctgatgc ccggccaggg cgccatcatc    840

ggcgtgggcg ccatggaata ccccgccgag tttcaaggcg ccagcgagga acgcatcgcc    900

gagctgggca tcggcaaatt gatcactttg acctccacct acgaccaccg catcatccag    960

ggcgcggaat cgggcgactt cctgcgcacc atccacgagt tgctgctctc ggatggcttc   1020

tgggacgagg tcttccgcga actgagcatc ccatatctgc cggtgcgctg gagcaccgac   1080

aaccccgact cgatcgtcga caagaacgct cgcgtcatga acttgatcgc ggcctaccgc   1140

aaccgcggcc atctgatggc cgataccgac ccgctgcggt tggacaaagc tcggttccgc   1200

agtcaccccg acctcgaagt gctgacccac ggcctgacgc tgtgggatct cgatcgggtg   1260

ttcaaggtcg acggctttgc cggtgcgcag tacaagaaac tgcgcgacgt gctgggcttg   1320

ctgcgcgatg cctactgccg ccacatcggc gtggagtacg cccatatcct cgaccccgaa   1380

caaaaggagt ggctcgaaca acgggtcgag accaagcacg tcaaacccac tgtggcccaa   1440

cagaaataca tcctcagcaa gctcaacgcc gccgaggcct ttgaaacgtt cctacagacc   1500

aagtacgtcg gccagaagcg gttctcgctg gaaggcgccg aaagcgtgat cccgatgatg   1560

gacgcggcga tcgaccagtg cgctgagcac ggcctcgacg aggtggtcat cgggatgccg   1620

caccggggcc ggctcaacgt gctggccaac atcgtcggca agccgtactc gcagatcttc   1680

accgagttcg agggcaacct gaatccgtcg caggcgcacg gctccggtga cgtcaagtac   1740

cacctgggcg ccaccgggct gtacctgcag atgttcggcg acaacgacat tcaggtgtcg   1800

ctgaccgcca acccgtcgca tctggaggcc gtcgacccgg tgctggaggg attggtgcgg   1860
```

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```
gccaagcagg atctgctcga ccacggaagc atcgacagcg acggccaacg ggcgttctcg   1920

gtggtgccgc tgatgttgca tggcgatgcc gcgttcgccg gtcagggtgt ggtcgccgag   1980

acgctgaacc tggcgaatct gccgggctac cgcgtcggcg gcaccatcca catcatcgtc   2040

aacaaccaga tcggcttcac caccgcgccc gagtattcca ggtccagcga gtactgcacc   2100

gacgtcgcaa agatgatcgg ggcaccgatc tttcacgtca acggcgacga cccggaggcg   2160

tgtgtctggg tggcgcggtt ggcggtggac ttccgacaac ggttcaagaa ggacgtcgtc   2220

atcgacatgc tgtgctaccg ccgccgcggg cacaacgagg gtgacgaccc gtcgatgacc   2280

aacccctaca tgtacgacgt cgtcgacacc aagcgcgggg cccgcaaaag ctacaccgaa   2340

gccctgatcg gacgtggcga catctcgatg aaggaggccg aggacgcgct gcgcgactac   2400

cagggccagc tggaacgggt gttcaacgaa gtgcgcgagc tggagaagca cggtgtgcag   2460

ccgagcgagt cggtcgagtc cgaccagatg attcccgcgg ggctggccac tgcggtggac   2520

aagtcgctgc tggcccggat cggcgatgcg ttcctcgcct tgccgaacgg cttcaccgcg   2580

cacccgcgag tccaaccggt gctggagaag cgccgggaga tggcctatga aggcaagatc   2640

gactgggcct ttggcgagct gctggcgctg ggctcgctgg tggccgaagg caagctggtg   2700

cgcttgtcgg ggcaggacag ccgccgcggc accttctccc agcggcattc ggttctcatc   2760

gaccgccaca ctggcgagga gttcacacca ctgcagctgc tggcgaccaa ctccgacggc   2820

agcccgaccg gcggaaagtt cctggtctac gactcgccac tgtcggagta cgccgccgtc   2880

ggcttcgagt acggctacac tgtgggcaat ccggacgccg tggtgctctg ggaggcgcag   2940

ttcggcgact tcgtcaacgg cgcacagtcg atcatcgacg agttcatcag ctccggtgag   3000

gccaagtggg gccaattgtc caacgtcgtg ctgctgttac cgcacgggca cgaggggcag   3060

ggacccgacc acacttctgc ccggatcgaa cgcttcttgc agttgtgggc ggaaggttcg   3120

atgaccatcg cgatgccgtc gactccgtcg aactacttcc acctgctacg ccggcatgcc   3180

ctggacggca tccaacgccc gctgatcgtg ttcacgccca agtcgatgtt gcgtcacaag   3240

gccgccgtca gcgaaatcaa ggacttcacc gagatcaagt tccgctcagt gctggaggaa   3300

cccacctatg aggacggcat cggagaccgc aacaaggtca gccggatcct gctgaccagt   3360

ggcaagctgt attacgagct ggccgcccgc aaggccaagg acaaccgcaa tgacctcgcg   3420

atcgtgcggc ttgaacagct cgccccgctg cccaggcgtc gactgcgtga aacgctggac   3480

cgctacgaga acgtcaagga gttcttctgg gtccaagagg aaccggccaa ccagggtgcg   3540

tggccgcgat tcgggctcga actacccgag ctgctgcctg acaagttggc cgggatcaag   3600

cgaatctcgc gccgggcgat gtcagccccg tcgtcaggct cgtcgaaggt gcacgccgtc   3660

gaacagcagg agatcctcga cgaggcgttc ggctaa                              3696
```

<210> SEQ ID NO 49
<211> LENGTH: 1231
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium bovis

<400> SEQUENCE: 49

```
Met Ala Asn Ile Ser Ser Pro Phe Gly Gln Asn Glu Trp Leu Val Glu
1               5                   10                  15

Glu Met Tyr Arg Lys Phe Arg Asp Asp Pro Ser Ser Val Asp Pro Ser
            20                  25                  30

Trp His Glu Phe Leu Val Asp Tyr Ser Pro Glu Pro Thr Ser Gln Pro
        35                  40                  45
```

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```
Ala Ala Glu Pro Thr Arg Val Thr Ser Pro Leu Val Ala Glu Arg Ala
    50                  55                  60

Ala Ala Ala Ala Pro Gln Ala Pro Pro Lys Pro Ala Asp Thr Ala Ala
65                  70                  75                  80

Ala Gly Asn Gly Val Val Ala Ala Leu Ala Ala Lys Thr Ala Val Pro
                85                  90                  95

Pro Pro Ala Glu Gly Asp Glu Val Ala Val Leu Arg Gly Ala Ala Ala
            100                 105                 110

Ala Val Val Lys Asn Met Ser Ala Ser Leu Glu Val Pro Thr Ala Thr
        115                 120                 125

Ser Val Arg Ala Val Pro Ala Lys Leu Leu Ile Asp Asn Arg Ile Val
    130                 135                 140

Ile Asn Asn Gln Leu Lys Arg Thr Arg Gly Gly Lys Ile Ser Phe Thr
145                 150                 155                 160

His Leu Leu Gly Tyr Ala Leu Val Gln Ala Val Lys Lys Phe Pro Asn
                165                 170                 175

Met Asn Arg His Tyr Thr Glu Val Asp Gly Lys Pro Thr Ala Val Thr
            180                 185                 190

Pro Ala His Thr Asn Leu Gly Leu Ala Ile Asp Leu Gln Gly Lys Asp
        195                 200                 205

Gly Lys Arg Ser Leu Val Val Ala Gly Ile Lys Arg Cys Glu Thr Met
    210                 215                 220

Arg Phe Ala Gln Phe Val Thr Ala Tyr Glu Asp Ile Val Arg Arg Ala
225                 230                 235                 240

Arg Asp Gly Lys Leu Thr Thr Glu Asp Phe Ala Gly Val Thr Ile Ser
                245                 250                 255

Leu Thr Asn Pro Gly Thr Ile Gly Thr Val His Ser Val Pro Arg Leu
            260                 265                 270

Met Pro Gly Gln Gly Ala Ile Ile Gly Val Gly Ala Met Glu Tyr Pro
        275                 280                 285

Ala Glu Phe Gln Gly Ala Ser Glu Glu Arg Ile Ala Glu Leu Gly Ile
    290                 295                 300

Gly Lys Leu Ile Thr Leu Thr Ser Thr Tyr Asp His Arg Ile Ile Gln
305                 310                 315                 320

Gly Ala Glu Ser Gly Asp Phe Leu Arg Thr Ile His Glu Leu Leu Leu
                325                 330                 335

Ser Asp Gly Phe Trp Asp Glu Val Phe Arg Glu Leu Ser Ile Pro Tyr
            340                 345                 350

Leu Pro Val Arg Trp Ser Thr Asp Asn Pro Asp Ser Ile Val Asp Lys
        355                 360                 365

Asn Ala Arg Val Met Asn Leu Ile Ala Ala Tyr Arg Asn Arg Gly His
    370                 375                 380

Leu Met Ala Asp Thr Asp Pro Leu Arg Leu Asp Lys Ala Arg Phe Arg
385                 390                 395                 400

Ser His Pro Asp Leu Glu Val Leu Thr His Gly Leu Thr Leu Trp Asp
                405                 410                 415

Leu Asp Arg Val Phe Lys Val Asp Gly Phe Ala Gly Ala Gln Tyr Lys
            420                 425                 430

Lys Leu Arg Asp Val Leu Gly Leu Leu Arg Asp Ala Tyr Cys Arg His
        435                 440                 445

Ile Gly Val Glu Tyr Ala His Ile Leu Asp Pro Glu Gln Lys Glu Trp
    450                 455                 460
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```
Leu Glu Gln Arg Val Glu Thr Lys His Val Lys Pro Thr Val Ala Gln
465                 470                 475                 480

Gln Lys Tyr Ile Leu Ser Lys Leu Asn Ala Ala Glu Ala Phe Glu Thr
                485                 490                 495

Phe Leu Gln Thr Lys Tyr Val Gly Gln Lys Arg Phe Ser Leu Glu Gly
            500                 505                 510

Ala Glu Ser Val Ile Pro Met Met Asp Ala Ala Ile Asp Gln Cys Ala
        515                 520                 525

Glu His Gly Leu Asp Glu Val Val Ile Gly Met Pro His Arg Gly Arg
    530                 535                 540

Leu Asn Val Leu Ala Asn Ile Val Gly Lys Pro Tyr Ser Gln Ile Phe
545                 550                 555                 560

Thr Glu Phe Glu Gly Asn Leu Asn Pro Ser Gln Ala His Gly Ser Gly
                565                 570                 575

Asp Val Lys Tyr His Leu Gly Ala Thr Gly Leu Tyr Leu Gln Met Phe
            580                 585                 590

Gly Asp Asn Asp Ile Gln Val Ser Leu Thr Ala Asn Pro Ser His Leu
        595                 600                 605

Glu Ala Val Asp Pro Val Leu Glu Gly Leu Val Arg Ala Lys Gln Asp
    610                 615                 620

Leu Leu Asp His Gly Ser Ile Asp Ser Asp Gly Gln Arg Ala Phe Ser
625                 630                 635                 640

Val Val Pro Leu Met Leu His Gly Asp Ala Ala Phe Ala Gly Gln Gly
                645                 650                 655

Val Val Ala Glu Thr Leu Asn Leu Ala Asn Leu Pro Gly Tyr Arg Val
            660                 665                 670

Gly Gly Thr Ile His Ile Ile Val Asn Asn Gln Ile Gly Phe Thr Thr
        675                 680                 685

Ala Pro Glu Tyr Ser Arg Ser Ser Glu Tyr Cys Thr Asp Val Ala Lys
    690                 695                 700

Met Ile Gly Ala Pro Ile Phe His Val Asn Gly Asp Asp Pro Glu Ala
705                 710                 715                 720

Cys Val Trp Val Ala Arg Leu Ala Val Asp Phe Arg Gln Arg Phe Lys
                725                 730                 735

Lys Asp Val Val Ile Asp Met Leu Cys Tyr Arg Arg Arg Gly His Asn
            740                 745                 750

Glu Gly Asp Asp Pro Ser Met Thr Asn Pro Tyr Met Tyr Asp Val Val
        755                 760                 765

Asp Thr Lys Arg Gly Ala Arg Lys Ser Tyr Thr Glu Ala Leu Ile Gly
    770                 775                 780

Arg Gly Asp Ile Ser Met Lys Glu Ala Glu Asp Ala Leu Arg Asp Tyr
785                 790                 795                 800

Gln Gly Gln Leu Glu Arg Val Phe Asn Glu Val Arg Glu Leu Glu Lys
                805                 810                 815

His Gly Val Gln Pro Ser Glu Ser Val Glu Ser Asp Gln Met Ile Pro
            820                 825                 830

Ala Gly Leu Ala Thr Ala Val Asp Lys Ser Leu Leu Ala Arg Ile Gly
        835                 840                 845

Asp Ala Phe Leu Ala Leu Pro Asn Gly Phe Thr Ala His Pro Arg Val
    850                 855                 860

Gln Pro Val Leu Glu Lys Arg Arg Glu Met Ala Tyr Glu Gly Lys Ile
865                 870                 875                 880

Asp Trp Ala Phe Gly Glu Leu Leu Ala Leu Gly Ser Leu Val Ala Glu
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               885                 890                 895

Gly Lys Leu Val Arg Leu Ser Gly Gln Asp Ser Arg Arg Gly Thr Phe
           900                 905                 910

Ser Gln Arg His Ser Val Leu Ile Asp Arg His Thr Gly Glu Glu Phe
       915                 920                 925

Thr Pro Leu Gln Leu Leu Ala Thr Asn Ser Asp Gly Ser Pro Thr Gly
   930                 935                 940

Gly Lys Phe Leu Val Tyr Asp Ser Pro Leu Ser Glu Tyr Ala Ala Val
945                 950                 955                 960

Gly Phe Glu Tyr Gly Tyr Thr Val Gly Asn Pro Asp Ala Val Val Leu
               965                 970                 975

Trp Glu Ala Gln Phe Gly Asp Phe Val Asn Gly Ala Gln Ser Ile Ile
           980                 985                 990

Asp Glu Phe Ile Ser Ser Gly Glu  Ala Lys Trp Gly Gln  Leu Ser Asn
       995                 1000                1005

Val Val  Leu Leu Leu Pro His  Gly His Glu Gly Gln  Gly Pro Asp
   1010                1015                1020

His Thr  Ser Ala Arg Ile Glu  Arg Phe Leu Gln Leu  Trp Ala Glu
   1025                1030                1035

Gly Ser  Met Thr Ile Ala Met  Pro Ser Thr Pro Ser  Asn Tyr Phe
   1040                1045                1050

His Leu  Leu Arg Arg His Ala  Leu Asp Gly Ile Gln  Arg Pro Leu
   1055                1060                1065

Ile Val  Phe Thr Pro Lys Ser  Met Leu Arg His Lys  Ala Ala Val
   1070                1075                1080

Ser Glu  Ile Lys Asp Phe Thr  Glu Ile Lys Phe Arg  Ser Val Leu
   1085                1090                1095

Glu Glu  Pro Thr Tyr Glu Asp  Gly Ile Gly Asp Arg  Asn Lys Val
   1100                1105                1110

Ser Arg  Ile Leu Leu Thr Ser  Gly Lys Leu Tyr Tyr  Glu Leu Ala
   1115                1120                1125

Ala Arg  Lys Ala Lys Asp Asn  Arg Asn Asp Leu Ala  Ile Val Arg
   1130                1135                1140

Leu Glu  Gln Leu Ala Pro Leu  Pro Arg Arg Arg Leu  Arg Glu Thr
   1145                1150                1155

Leu Asp  Arg Tyr Glu Asn Val  Lys Glu Phe Phe Trp  Val Gln Glu
   1160                1165                1170

Glu Pro  Ala Asn Gln Gly Ala  Trp Pro Arg Phe Gly  Leu Glu Leu
   1175                1180                1185

Pro Glu  Leu Leu Pro Asp Lys  Leu Ala Gly Ile Lys  Arg Ile Ser
   1190                1195                1200

Arg Arg  Ala Met Ser Ala Pro  Ser Ser Gly Ser Ser  Lys Val His
   1205                1210                1215

Ala Val  Glu Gln Gln Glu Ile  Leu Asp Glu Ala Phe  Gly
   1220                1225                1230


<210> SEQ ID NO 50
<211> LENGTH: 1356
<212> TYPE: DNA
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 50

atggaaatca aagaaatggt gagccttgca cgcaaggctc agaaggagta tcaagctacc     60

cataaccaag aagcagttga caacatttgc cgagctgcag caaaagttat ttatgaaaat    120
```

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```
gcagctattc tggctcgcga agcagtagac gaaaccggca tgggcgttta cgaacacaaa    180

gtggccaaga atcaaggcaa atccaaaggt gtttggtaca acctccacaa taaaaaatcg    240

attggtatcc tcaatataga cgagcgtacc ggtatgatcg agattgcaaa gcctatcgga    300

gttgtaggag ccgtaacgcc gacgaccaac ccgatcgtta ctccgatgag caatatcatc    360

tttgctctta agacctgcaa tgccatcatt attgcccccc accccagatc caaaaaatgc    420

tctgcacacg cagttcgtct gatcaaagaa gctatcgctc cgttcaacgt accggaaggt    480

atggttcaga tcatcgaaga acccagcatc gagaagacgc aggaactcat gggcgccgta    540

gacgtagtag ttgctacggg tggtatgggc atggtgaagt ctgcatattc ttcaggaaag    600

ccttctttcg gtgttggagc cggtaacgtt caggtgatcg tggatagcaa catcgatttc    660

gaagctgctg cagaaaaaat catcaccggt cgtgctttcg acaacggtat catctgctca    720

ggcgaacaga gcatcatcta caacgaggct gacaaggaag cagttttcac agcattccgc    780

aaccacggtg catatttctg tgacgaagcc gaaggagatc gggctcgtgc agctatcttc    840

gaaaatggag ccatcgcgaa agatgtagta ggtcagagcg ttgccttcat tgccaagaaa    900

gcaaacatca atatccccga gggtacccgt attctcgttg ttgaagctcg cggcgtagga    960

gcagaagacg ttatctgtaa ggaaaagatg tgtcccgtaa tgtgcgccct cagctacaag   1020

cacttcgaag aaggtgtaga aatcgcacgt acgaacctcg ccaacgaagg taacggccac   1080

acctgtgcta tccactccaa caatcaggca cacatcatcc tcgcaggatc agagctgacg   1140

gtatctcgta tcgtagtgaa tgctccgagt gccactacag caggcggtca catccaaaac   1200

ggtcttgccg taaccaatac gctcggatgc ggatcatggg gtaataactc tatctccgag   1260

aacttcactt acaagcacct cctcaacatt tcacgcatcg caccgttgaa ttcaagcatt   1320

cacatccccg atgacaaaga aatctgggaa ctctaa                             1356
```

<210> SEQ ID NO 51
<211> LENGTH: 451
<212> TYPE: PRT
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 51

```
Met Glu Ile Lys Glu Met Val Ser Leu Ala Arg Lys Ala Gln Lys Glu
1               5                   10                  15

Tyr Gln Ala Thr His Asn Gln Glu Ala Val Asp Asn Ile Cys Arg Ala
            20                  25                  30

Ala Ala Lys Val Ile Tyr Glu Asn Ala Ala Ile Leu Ala Arg Glu Ala
        35                  40                  45

Val Asp Glu Thr Gly Met Gly Val Tyr Glu His Lys Val Ala Lys Asn
    50                  55                  60

Gln Gly Lys Ser Lys Gly Val Trp Tyr Asn Leu His Asn Lys Lys Ser
65                  70                  75                  80

Ile Gly Ile Leu Asn Ile Asp Glu Arg Thr Gly Met Ile Glu Ile Ala
                85                  90                  95

Lys Pro Ile Gly Val Val Gly Ala Val Thr Pro Thr Thr Asn Pro Ile
            100                 105                 110

Val Thr Pro Met Ser Asn Ile Ile Phe Ala Leu Lys Thr Cys Asn Ala
        115                 120                 125

Ile Ile Ile Ala Pro His Pro Arg Ser Lys Lys Cys Ser Ala His Ala
    130                 135                 140

Val Arg Leu Ile Lys Glu Ala Ile Ala Pro Phe Asn Val Pro Glu Gly
```

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```
145                 150                 155                 160

Met Val Gln Ile Ile Glu Glu Pro Ser Ile Glu Lys Thr Gln Glu Leu
                165                 170                 175

Met Gly Ala Val Asp Val Val Val Ala Thr Gly Gly Met Gly Met Val
            180                 185                 190

Lys Ser Ala Tyr Ser Ser Gly Lys Pro Ser Phe Gly Val Gly Ala Gly
        195                 200                 205

Asn Val Gln Val Ile Val Asp Ser Asn Ile Asp Phe Glu Ala Ala Ala
    210                 215                 220

Glu Lys Ile Ile Thr Gly Arg Ala Phe Asp Asn Gly Ile Ile Cys Ser
225                 230                 235                 240

Gly Glu Gln Ser Ile Ile Tyr Asn Glu Ala Asp Lys Glu Ala Val Phe
                245                 250                 255

Thr Ala Phe Arg Asn His Gly Ala Tyr Phe Cys Asp Glu Ala Glu Gly
            260                 265                 270

Asp Arg Ala Arg Ala Ala Ile Phe Glu Asn Gly Ala Ile Ala Lys Asp
        275                 280                 285

Val Val Gly Gln Ser Val Ala Phe Ile Ala Lys Lys Ala Asn Ile Asn
    290                 295                 300

Ile Pro Glu Gly Thr Arg Ile Leu Val Val Glu Ala Arg Gly Val Gly
305                 310                 315                 320

Ala Glu Asp Val Ile Cys Lys Glu Lys Met Cys Pro Val Met Cys Ala
                325                 330                 335

Leu Ser Tyr Lys His Phe Glu Glu Gly Val Glu Ile Ala Arg Thr Asn
            340                 345                 350

Leu Ala Asn Glu Gly Asn Gly His Thr Cys Ala Ile His Ser Asn Asn
        355                 360                 365

Gln Ala His Ile Ile Leu Ala Gly Ser Glu Leu Thr Val Ser Arg Ile
    370                 375                 380

Val Val Asn Ala Pro Ser Ala Thr Thr Ala Gly Gly His Ile Gln Asn
385                 390                 395                 400

Gly Leu Ala Val Thr Asn Thr Leu Gly Cys Gly Ser Trp Gly Asn Asn
                405                 410                 415

Ser Ile Ser Glu Asn Phe Thr Tyr Lys His Leu Leu Asn Ile Ser Arg
            420                 425                 430

Ile Ala Pro Leu Asn Ser Ser Ile His Ile Pro Asp Asp Lys Glu Ile
        435                 440                 445

Trp Glu Leu
    450
```

<210> SEQ ID NO 52
<211> LENGTH: 1116
<212> TYPE: DNA
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 52

```
atgcaacttt tcaaactcaa gagtgtaaca catcactttg acacttttgc agaatttgcc     60

aaggaattct gtcttggaga acgcgacttg gtaattacca acgagttcat ctatgaaccg    120

tatatgaagg catgccagct cccctgccat tttgttatgc aggagaaata tgggcaaggc    180

gagccttctg acgaaatgat gaataacatc ttggcagaca tccgtaatat ccagttcgac    240

cgcgtaatcg gtatcggagg aggtacggtt attgacatct ctaaactttt cgttctgaaa    300

ggattaaatg atgtactcga tgcattcgac cgcaaaatac ctcttatcaa agagaaagaa    360
```

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```
ctgatcattg tgcccacaac atgcggaacg ggtagcgagg tgacgaacat ttctatcgca    420

gaaatcaaaa gccgtcacac caaaatggga ttggctgacg atgccattgt tgcagaccat    480

gccatcatca tacctgaact tctgaagagc ttgcctttcc acttctacgc atgcagtgca    540

atcgatgctc ttatccatgc catcgagtca tacgtatctc ctaaagccag tccatattct    600

cgtctgttca gtgaggcggc ttgggacatt atcctggaag tattcaagaa aatcgccgaa    660

cacggccctg aataccgctt cgaaaagctg ggagaaatga tcatggccag caactatgcc    720

ggtatagcct tcggaaatgc aggagtagga gccgtccacg cactatccta cccgttggga    780

ggcaactatc acgtgccgca tggagaagca aactatcagt tcttcacaga ggtattcaaa    840

gtataccaaa agaagaatcc tttcggctat atagtcgaac tcaactggaa gctctccaag    900

atactgaact gccagcccga atacgtatat ccgaagctgg atgaacttct cggatgcctt    960

cttaccaaga aacctttgca cgaatacggc atgaaggacg aagaggtaag aggctttgcg   1020

gaatcagtgc ttaagacaca gcaaagattg ctcgccaaca actacgtaga gcttactgta   1080

gatgagatcg aaggtatcta cagaagactc tactaa                             1116
```

<210> SEQ ID NO 53
<211> LENGTH: 371
<212> TYPE: PRT
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 53

```
Met Gln Leu Phe Lys Leu Lys Ser Val Thr His His Phe Asp Thr Phe
1               5                   10                  15

Ala Glu Phe Ala Lys Glu Phe Cys Leu Gly Glu Arg Asp Leu Val Ile
            20                  25                  30

Thr Asn Glu Phe Ile Tyr Glu Pro Tyr Met Lys Ala Cys Gln Leu Pro
        35                  40                  45

Cys His Phe Val Met Gln Glu Lys Tyr Gly Gln Gly Glu Pro Ser Asp
    50                  55                  60

Glu Met Met Asn Asn Ile Leu Ala Asp Ile Arg Asn Ile Gln Phe Asp
65                  70                  75                  80

Arg Val Ile Gly Ile Gly Gly Gly Thr Val Ile Asp Ile Ser Lys Leu
                85                  90                  95

Phe Val Leu Lys Gly Leu Asn Asp Val Leu Asp Ala Phe Asp Arg Lys
            100                 105                 110

Ile Pro Leu Ile Lys Glu Lys Glu Leu Ile Ile Val Pro Thr Thr Cys
        115                 120                 125

Gly Thr Gly Ser Glu Val Thr Asn Ile Ser Ile Ala Glu Ile Lys Ser
    130                 135                 140

Arg His Thr Lys Met Gly Leu Ala Asp Asp Ala Ile Val Ala Asp His
145                 150                 155                 160

Ala Ile Ile Ile Pro Glu Leu Leu Lys Ser Leu Pro Phe His Phe Tyr
                165                 170                 175

Ala Cys Ser Ala Ile Asp Ala Leu Ile His Ala Ile Glu Ser Tyr Val
            180                 185                 190

Ser Pro Lys Ala Ser Pro Tyr Ser Arg Leu Phe Ser Glu Ala Ala Trp
        195                 200                 205

Asp Ile Ile Leu Glu Val Phe Lys Lys Ile Ala Glu His Gly Pro Glu
    210                 215                 220

Tyr Arg Phe Glu Lys Leu Gly Glu Met Ile Met Ala Ser Asn Tyr Ala
225                 230                 235                 240
```

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```
Gly Ile Ala Phe Gly Asn Ala Gly Val Gly Ala Val His Ala Leu Ser
                245                 250                 255

Tyr Pro Leu Gly Gly Asn Tyr His Val Pro His Gly Glu Ala Asn Tyr
            260                 265                 270

Gln Phe Phe Thr Glu Val Phe Lys Val Tyr Gln Lys Lys Asn Pro Phe
        275                 280                 285

Gly Tyr Ile Val Glu Leu Asn Trp Lys Leu Ser Lys Ile Leu Asn Cys
    290                 295                 300

Gln Pro Glu Tyr Val Tyr Pro Lys Leu Asp Glu Leu Leu Gly Cys Leu
305                 310                 315                 320

Leu Thr Lys Lys Pro Leu His Glu Tyr Gly Met Lys Asp Glu Glu Val
                325                 330                 335

Arg Gly Phe Ala Glu Ser Val Leu Lys Thr Gln Gln Arg Leu Leu Ala
            340                 345                 350

Asn Asn Tyr Val Glu Leu Thr Val Asp Glu Ile Glu Gly Ile Tyr Arg
        355                 360                 365

Arg Leu Tyr
    370
```

<210> SEQ ID NO 54
<211> LENGTH: 1296
<212> TYPE: DNA
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 54

```
atgaaagacg tattagcgga atatgcctcc cgaattgttt cggccgaaga agccgtaaaa     60

catatcaaaa atggagaacg ggtagctttg tcacatgctg ccggagttcc tcagagttgt    120

gttgatgcac tggtacaaca ggccgacctt ttccagaatg tcgaaattta tcacatgctt    180

tgtctcggcg aaggaaaata tatggcacct gaaatggccc ctcacttccg acacataacc    240

aattttgtag gtggtaattc tcgtaaagca gttgaggaaa atagagccga cttcattccg    300

gtattctttt atgaagtgcc atcaatgatt cgcaaagaca tccttcacat agatgtcgcc    360

atcgttcagc tttcaatgcc tgatgagaat ggttactgta gttttggagt atcttgcgat    420

tatagcaaac cggcagcaga aagcgctcat ttagttatag gggaaatcaa ccgtcaaatg    480

ccatatgtac atggcgacaa cttgattcac atatcgaagt tggattacat cgtgatggca    540

gactacccta tctattctct tgcaaagccc aaaatcggag aagtagaaga agctatcggg    600

cgtaattgtg ccgagcttat tgaagatggt gccacactcc aactcggtat cggcgcgatt    660

cctgatgcag ccctgttatt cctcaaggac aaaaaagatc tggggatcca taccgagatg    720

ttctccgatg gtgttgtcga attagttcgc agtggagtaa ttacaggaaa gaaaaagaca    780

cttcaccccg gaaagatggt cgcaaccttc ttaatgggaa gcgaagacgt atatcatttc    840

atcgacaaaa atcccgatgt agaactttat ccggtagatt acgtcaatga tccgcgagta    900

atcgctcaaa atgataatat ggtcagcatc aatagctgta tcgaaatcga tcttatggga    960

caagtcgtgt ccgaatgtat aggaagcaag caattcagcg gaaccggcgg tcaagtagat   1020

tatgttcgtg gagcagcatg gtctaaaaac ggcaaaagca tcatggcaat tccctcaaca   1080

gccaaaaacg gtactgcatc tcgaattgta cctataattg cagagggagc tgctgtaaca   1140

accctccgca acgaagtcga ttacgttgta accgaatacg gtatagcaca actcaaagga   1200

aagagtttgc gccagcgagc agaagctctt attgccatag cccacccgga tttcagagag   1260

gaactaacga aacatctccg caaacgtttc ggataa                              1296
```

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<210> SEQ ID NO 55
<211> LENGTH: 431
<212> TYPE: PRT
<213> ORGANISM: Porphyromonas gingivalis

<400> SEQUENCE: 55

Met Lys Asp Val Leu Ala Glu Tyr Ala Ser Arg Ile Val Ser Ala Glu
1 5 10 15

Glu Ala Val Lys His Ile Lys Asn Gly Glu Arg Val Ala Leu Ser His
20 25 30

Ala Ala Gly Val Pro Gln Ser Cys Val Asp Ala Leu Val Gln Gln Ala
35 40 45

Asp Leu Phe Gln Asn Val Glu Ile Tyr His Met Leu Cys Leu Gly Glu
50 55 60

Gly Lys Tyr Met Ala Pro Glu Met Ala Pro His Phe Arg His Ile Thr
65 70 75 80

Asn Phe Val Gly Gly Asn Ser Arg Lys Ala Val Glu Glu Asn Arg Ala
85 90 95

Asp Phe Ile Pro Val Phe Phe Tyr Glu Val Pro Ser Met Ile Arg Lys
100 105 110

Asp Ile Leu His Ile Asp Val Ala Ile Val Gln Leu Ser Met Pro Asp
115 120 125

Glu Asn Gly Tyr Cys Ser Phe Gly Val Ser Cys Asp Tyr Ser Lys Pro
130 135 140

Ala Ala Glu Ser Ala His Leu Val Ile Gly Glu Ile Asn Arg Gln Met
145 150 155 160

Pro Tyr Val His Gly Asp Asn Leu Ile His Ile Ser Lys Leu Asp Tyr
165 170 175

Ile Val Met Ala Asp Tyr Pro Ile Tyr Ser Leu Ala Lys Pro Lys Ile
180 185 190

Gly Glu Val Glu Glu Ala Ile Gly Arg Asn Cys Ala Glu Leu Ile Glu
195 200 205

Asp Gly Ala Thr Leu Gln Leu Gly Ile Gly Ala Ile Pro Asp Ala Ala
210 215 220

Leu Leu Phe Leu Lys Asp Lys Lys Asp Leu Gly Ile His Thr Glu Met
225 230 235 240

Phe Ser Asp Gly Val Val Glu Leu Val Arg Ser Gly Val Ile Thr Gly
245 250 255

Lys Lys Lys Thr Leu His Pro Gly Lys Met Val Ala Thr Phe Leu Met
260 265 270

Gly Ser Glu Asp Val Tyr His Phe Ile Asp Lys Asn Pro Asp Val Glu
275 280 285

Leu Tyr Pro Val Asp Tyr Val Asn Asp Pro Arg Val Ile Ala Gln Asn
290 295 300

Asp Asn Met Val Ser Ile Asn Ser Cys Ile Glu Ile Asp Leu Met Gly
305 310 315 320

Gln Val Val Ser Glu Cys Ile Gly Ser Lys Gln Phe Ser Gly Thr Gly
325 330 335

Gly Gln Val Asp Tyr Val Arg Gly Ala Ala Trp Ser Lys Asn Gly Lys
340 345 350

Ser Ile Met Ala Ile Pro Ser Thr Ala Lys Asn Gly Thr Ala Ser Arg
355 360 365

Ile Val Pro Ile Ile Ala Glu Gly Ala Ala Val Thr Thr Leu Arg Asn
370 375 380

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Glu Val Asp Tyr Val Val Thr Glu Tyr Gly Ile Ala Gln Leu Lys Gly
385                 390                 395                 400

Lys Ser Leu Arg Gln Arg Ala Glu Ala Leu Ile Ala Ile Ala His Pro
                405                 410                 415

Asp Phe Arg Glu Glu Leu Thr Lys His Leu Arg Lys Arg Phe Gly
            420                 425                 430


<210> SEQ ID NO 56
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Clostridium acetobutylicum

<400> SEQUENCE: 56

atgattaaga gttttaatga aattatcatg aaggtaaaga gcaaagaaat gaaaaaagtt     60

gctgttgctg tagcacaaga cgagccagta cttgaagcag taagagatgc taagaaaaat    120

ggtattgcag atgctattct tgttggagac catgacgaaa tcgtgtcaat cgcgcttaaa    180

ataggaatgg atgtaaatga ttttgaaata gtaaacgagc ctaacgttaa gaaagctgct    240

ttaaaggcag tagagcttgt atcaactgga aaagctgata tggtaatgaa gggacttgta    300

aatacagcaa ctttcttaag atctgtatta aacaaagaag ttggacttag aacaggaaaa    360

actatgtctc acgttgcagt atttgaaact gagaaatttg atagactatt atttttaaca    420

gatgttgctt tcaatactta tcctgaatta aaggaaaaaa ttgatatagt aaacaattca    480

gttaaggttg cacatgcaat aggaattgaa aatccaaagg ttgctccaat ttgtgcagtt    540

gaggttataa accctaaaat gccatcaaca cttgatgcag caatgctttc aaaaatgagt    600

gacagaggac aaattaaagg ttgtgtagtt gacggacctt tagcacttga tatagcttta    660

tcagaagaag cagcacatca taagggagta acaggagaag ttgctggaaa agctgatatc    720

ttcttaatgc caaacataga aacaggaaat gtaatgtata agactttaac atatacaact    780

gattcaaaaa atggaggaat cttagttgga acttctgcac cagttgtttt aacttcaaga    840

gctgacagcc atgaaacaaa aatgaactct atagcacttg cagctttagt tgcaggcaat    900

aaataa                                                               906


<210> SEQ ID NO 57
<211> LENGTH: 301
<212> TYPE: PRT
<213> ORGANISM: Clostridium acetobutylicum

<400> SEQUENCE: 57

Met Ile Lys Ser Phe Asn Glu Ile Ile Met Lys Val Lys Ser Lys Glu
1               5                   10                  15

Met Lys Lys Val Ala Val Ala Val Ala Gln Asp Glu Pro Val Leu Glu
            20                  25                  30

Ala Val Arg Asp Ala Lys Lys Asn Gly Ile Ala Asp Ala Ile Leu Val
        35                  40                  45

Gly Asp His Asp Glu Ile Val Ser Ile Ala Leu Lys Ile Gly Met Asp
    50                  55                  60

Val Asn Asp Phe Glu Ile Val Asn Glu Pro Asn Val Lys Lys Ala Ala
65                  70                  75                  80

Leu Lys Ala Val Glu Leu Val Ser Thr Gly Lys Ala Asp Met Val Met
                85                  90                  95

Lys Gly Leu Val Asn Thr Ala Thr Phe Leu Arg Ser Val Leu Asn Lys
            100                 105                 110
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Glu Val Gly Leu Arg Thr Gly Lys Thr Met Ser His Val Ala Val Phe
        115                 120                 125

Glu Thr Glu Lys Phe Asp Arg Leu Leu Phe Leu Thr Asp Val Ala Phe
    130                 135                 140

Asn Thr Tyr Pro Glu Leu Lys Glu Lys Ile Asp Ile Val Asn Asn Ser
145                 150                 155                 160

Val Lys Val Ala His Ala Ile Gly Ile Glu Asn Pro Lys Val Ala Pro
                165                 170                 175

Ile Cys Ala Val Glu Val Ile Asn Pro Lys Met Pro Ser Thr Leu Asp
            180                 185                 190

Ala Ala Met Leu Ser Lys Met Ser Asp Arg Gly Gln Ile Lys Gly Cys
        195                 200                 205

Val Val Asp Gly Pro Leu Ala Leu Asp Ile Ala Leu Ser Glu Glu Ala
    210                 215                 220

Ala His His Lys Gly Val Thr Gly Glu Val Ala Gly Lys Ala Asp Ile
225                 230                 235                 240

Phe Leu Met Pro Asn Ile Glu Thr Gly Asn Val Met Tyr Lys Thr Leu
                245                 250                 255

Thr Tyr Thr Thr Asp Ser Lys Asn Gly Gly Ile Leu Val Gly Thr Ser
            260                 265                 270

Ala Pro Val Val Leu Thr Ser Arg Ala Asp Ser His Glu Thr Lys Met
        275                 280                 285

Asn Ser Ile Ala Leu Ala Ala Leu Val Ala Gly Asn Lys
    290                 295                 300


<210> SEQ ID NO 58
<211> LENGTH: 1068
<212> TYPE: DNA
<213> ORGANISM: Clostridium acetobutylicum

<400> SEQUENCE: 58

atgtatagat tactaataat caatcctggc tcgacctcaa ctaaaattgg tatttatgac     60

gatgaaaaag agatatttga gaagacttta agacattcag ctgaagagat agaaaaatat    120

aacactatat ttgatcaatt tcaattcaga aagaatgtaa ttttagatgc gttaaaagaa    180

gcaaacatag aagtaagttc tttaaatgct gtagttggaa gaggcggact cttaaagcca    240

atagtaagtg gaacttatgc agtaaatcaa aaaatgcttg aagaccttaa agtaggagtt    300

caaggtcagc atgcgtcaaa tcttggtgga attattgcaa atgaaatagc aaaagaaata    360

aatgttccag catacatagt tgatccagtt gttgtggatg agcttgatga agtttcaaga    420

atatcaggaa tggctgacat tccaagaaaa agtatattcc atgcattaaa tcaaaaagca    480

gttgctagaa gatatgcaaa agaagttgga aaaaaatacg aagatcttaa tttaatcgta    540

gtccacatgg gtggaggtac ttcagtaggt actcataaag atggtagagt aatagaagtt    600

aataatacac ttgatggaga aggtccattc tcaccagaaa gaagtggtgg agttccaata    660

ggagatcttg taagattgtg cttcagcaac aaatatactt atgaagaagt aatgaaaaag    720

ataaacggca aaggcggagt tgttagttac ttaaatacta tcgattttaa ggctgtagtt    780

gataaagctc ttgaaggaga taagaaatgt gcacttatat atgaagcttt cacattccag    840

gtagcaaaag agataggaaa atgttcaacc gttttaaaag gaaatgtaga tgcaataatc    900

ttaacaggcg gaattgcgta caacgagcat gtatgtaatg ccatagagga tagagtaaaa    960

ttcatagcac ctgtagttag atatggtgga gaagatgaac ttcttgcact tgcagaaggt   1020

ggacttagag ttttaagagg agaagaaaaa gctaaggaat acaaataa                1068
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-continued

<210> SEQ ID NO 59
<211> LENGTH: 355
<212> TYPE: PRT
<213> ORGANISM: Clostridium acetobutylicum

<400> SEQUENCE: 59

Met Tyr Arg Leu Leu Ile Ile Asn Pro Gly Ser Thr Ser Thr Lys Ile
1 5 10 15

Gly Ile Tyr Asp Asp Glu Lys Glu Ile Phe Glu Lys Thr Leu Arg His
20 25 30

Ser Ala Glu Glu Ile Glu Lys Tyr Asn Thr Ile Phe Asp Gln Phe Gln
35 40 45

Phe Arg Lys Asn Val Ile Leu Asp Ala Leu Lys Glu Ala Asn Ile Glu
50 55 60

Val Ser Ser Leu Asn Ala Val Val Gly Arg Gly Gly Leu Leu Lys Pro
65 70 75 80

Ile Val Ser Gly Thr Tyr Ala Val Asn Gln Lys Met Leu Glu Asp Leu
85 90 95

Lys Val Gly Val Gln Gly Gln His Ala Ser Asn Leu Gly Gly Ile Ile
100 105 110

Ala Asn Glu Ile Ala Lys Glu Ile Asn Val Pro Ala Tyr Ile Val Asp
115 120 125

Pro Val Val Val Asp Glu Leu Asp Glu Val Ser Arg Ile Ser Gly Met
130 135 140

Ala Asp Ile Pro Arg Lys Ser Ile Phe His Ala Leu Asn Gln Lys Ala
145 150 155 160

Val Ala Arg Arg Tyr Ala Lys Glu Val Gly Lys Lys Tyr Glu Asp Leu
165 170 175

Asn Leu Ile Val Val His Met Gly Gly Gly Thr Ser Val Gly Thr His
180 185 190

Lys Asp Gly Arg Val Ile Glu Val Asn Asn Thr Leu Asp Gly Glu Gly
195 200 205

Pro Phe Ser Pro Glu Arg Ser Gly Gly Val Pro Ile Gly Asp Leu Val
210 215 220

Arg Leu Cys Phe Ser Asn Lys Tyr Thr Tyr Glu Glu Val Met Lys Lys
225 230 235 240

Ile Asn Gly Lys Gly Gly Val Val Ser Tyr Leu Asn Thr Ile Asp Phe
245 250 255

Lys Ala Val Val Asp Lys Ala Leu Glu Gly Asp Lys Lys Cys Ala Leu
260 265 270

Ile Tyr Glu Ala Phe Thr Phe Gln Val Ala Lys Glu Ile Gly Lys Cys
275 280 285

Ser Thr Val Leu Lys Gly Asn Val Asp Ala Ile Ile Leu Thr Gly Gly
290 295 300

Ile Ala Tyr Asn Glu His Val Cys Asn Ala Ile Glu Asp Arg Val Lys
305 310 315 320

Phe Ile Ala Pro Val Val Arg Tyr Gly Gly Glu Asp Glu Leu Leu Ala
325 330 335

Leu Ala Glu Gly Gly Leu Arg Val Leu Arg Gly Glu Glu Lys Ala Lys
340 345 350

Glu Tyr Lys
355

-continued

<210> SEQ ID NO 60
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 60

atgattaaga gttttaatga aattatcatg aaggtaaaga gcaaagaaat gaaaaaagtt 60

gctgttgctg tagcacaaga cgagccagta cttgaagcag tacgcgatgc taagaaaaat 120

ggtattgcag atgctattct tgttggcgac catgacgaaa tcgtgtcaat cgcgcttaaa 180

ataggcatgg atgtaaatga ttttgaaata gtaaacgagc ctaacgttaa gaaagctgct 240

ttaaaggcag tagagctggt atcaactgga aaagctgata tggtaatgaa gggacttgta 300

aatacagcaa ctttcttacg ctctgtatta aacaaagaag ttggactgag aacaggaaaa 360

actatgtctc acgttgcagt atttgaaact gagaaatttg atcgtctgtt atttttaaca 420

gatgttgctt tcaatactta tcctgaatta aaggaaaaaa ttgatatcgt aaacaattca 480

gttaaggttg cacatgcaat aggtattgaa aatccaaagg ttgctccaat ttgtgcagtt 540

gaggttataa accctaaaat gccatcaaca cttgatgcag caatgctttc aaaaatgagt 600

gacagaggac aaattaaagg ttgtgtagtt gacggaccgt tagcacttga tatcgcttta 660

tcagaagaag cagcacatca taagggcgta acaggagaag ttgctggaaa agctgatatc 720

ttcttaatgc caaacattga aacaggaaat gtaatgtata agactttaac atatacaact 780

gatagcaaaa atggcggaat cttagttgga acttctgcac cagttgtttt aacttcacgc 840

gctgacagcc atgaaacaaa aatgaactct attgcacttg cagctttagt tgcaggcaat 900

aaataa 906

<210> SEQ ID NO 61
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 61

atgattaaga gttttaatga aattatcatg aaggtaaaga gcaaagaaat gaaaaaagtt 60

gctgttgctg tagcacaaga cgagccagta cttgaagcag tacgcgatgc taagaaaaat 120

ggtattgccg atgctattct ggttggcgac catgacgaaa tcgtgtctat cgcgctgaaa 180

ataggcatgg atgtaaatga ttttgaaatt gttaacgagc ctaacgttaa gaaagctgcg 240

ttaaaggcag tagagctggt atcaactgga aaagctgata tggtaatgaa gggactggta 300

aataccgcaa ctttcttacg ctctgtatta aacaaagaag ttggtctgcg tacaggaaaa 360

accatgtctc acgttgcagt atttgaaact gagaaatttg atcgtctgtt atttttaaca 420

gatgttgctt tcaatactta tcctgaatta aaggaaaaaa ttgatatcgt taacaatagc 480

gttaaggttg cacatgccat tggtattgaa aatccaaagg ttgctccaat ttgtgcagtt 540

gaggttatta acccgaaaat gccatcaaca cttgatgcag caatgctttc aaaaatgagt 600

gaccgcggac aaattaaagg ttgtgtagtt gacggaccgc tggcacttga tatcgcttta 660

tcagaagaag cagcacatca taaaggcgta acaggagaag ttgctggaaa agctgatatc 720

ttcttaatgc caaacattga aacaggaaat gtaatgtata agacgttaac ctataccact 780

-continued

gatagcaaaa atggcggcat cctggttgga acttctgcac cagttgtttt aacttcacgc 840

gctgacagcc atgaaacaaa aatgaactct attgcactgg cagcgctggt tgcaggcaat 900

aaataa 906

<210> SEQ ID NO 62
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 62

atgattaaga gttttaatga aattatcatg aaggtaaaga gcaaagaaat gaaaaaagtt 60

gctgttgctg ttgcacaaga cgagccggta ctggaagcgg tacgcgatgc taagaaaaat 120

ggtattgccg atgctattct ggttggcgac catgacgaaa tcgtctctat cgcgctgaaa 180

attggcatgg atgttaatga ttttgaaatt gttaacgagc ctaacgttaa gaaagctgcg 240

ctgaaggcgg tagagctggt ttccaccgga aaagctgata tggtaatgaa agggctggtg 300

aataccgcaa ctttcttacg cagcgtactg aacaaagaag ttggtctgcg taccggaaaa 360

accatgagtc acgttgcggt atttgaaact gagaaatttg atcgtctgct gtttctgacc 420

gatgttgctt tcaatactta tcctgaatta aaagaaaaaa ttgatatcgt taacaatagc 480

gttaaggttg cgcatgccat tggtattgaa aatccaaagg ttgctccaat ttgtgcagtt 540

gaggttatta acccgaaaat gccatcaaca cttgatgccg caatgcttag caaaatgagt 600

gaccgcggac aaattaaagg ttgtgtggtt gacggcccgc tggcactgga tatcgcgtta 660

agcgaagaag cggcacatca taaaggcgta accggcgaag ttgctggaaa agctgatatc 720

ttcctgatgc caaacattga aacaggcaat gtaatgtata aaacgttaac ctataccact 780

gatagcaaaa atggcggcat cctggttgga acttctgcac cagttgtttt aacctcacgc 840

gctgacagcc atgaaaccaa aatgaacagc attgcactgg cagcgctggt tgcaggcaat 900

aaataa 906

<210> SEQ ID NO 63
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 63

atgattaaaa gttttaacga aattatcatg aaagtgaaaa gcaaagagat gaaaaaagtg 60

gcggttgcgg ttgcgcagga tgaaccggtg ctggaagcgg tgcgcgatgc caaaaaaaac 120

ggtattgccg atgccattct ggtgggcgat cacgatgaaa ttgtctctat tgcgctgaaa 180

attggcatgg atgttaacga ttttgaaatt gttaatgaac cgaacgtgaa aaaagcggcg 240

ctgaaagcgg ttgaactggt ttccaccggt aaagccgata tggtgatgaa agggctggtg 300

aataccgcaa ccttcctgcg cagcgtgctg aataaagaag tgggtctgcg taccggtaaa 360

accatgagtc atgttgcggt gtttgaaacc gaaaaatttg accgtctgct gtttctgacc 420

gatgttgcgt ttaataccta tccggaactg aaagagaaaa ttgatatcgt taataacagc 480

gtgaaagtgg cgcatgccat tggtattgaa aacccgaaag tggcgccgat ttgcgcggtt 540

gaagtgatta acccgaaaat gccgtcaacg ctggatgccg cgatgctcag caaaatgagc 600

-continued

gatcgcggtc aaatcaaagg ctgtgtggtt gatggcccgc tggcgctgga tatcgcgctt 660

agcgaagaag cggcgcatca taaaggcgtg accggcgaag tggccggtaa agccgatatt 720

ttcctgatgc cgaatattga aaccggcaac gtgatgtata aaacgctgac ctataccacc 780

gacagcaaaa acggcggcat tctggtgggt accagcgcgc cggtggtgct gacctcgcgc 840

gccgacagcc atgaaaccaa aatgaacagc attgcgctgg cggcgctggt ggccggtaat 900

aaataa 906

<210> SEQ ID NO 64
<211> LENGTH: 1068
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 64

atgtatcgtt tactgattat caatcctggc tcgacctcaa ctaaaattgg tatttatgac 60

gatgaaaaag agatatttga gaagacttta cgtcattcag ctgaagagat agaaaaatat 120

aacactatat ttgatcaatt tcagttcaga aagaatgtaa ttctcgatgc gttaaaagaa 180

gcaaacattg aagtaagttc tttaaatgct gtagttggac gcggcggact gttaaagcca 240

atagtaagtg gaacttatgc agtaaatcaa aaaatgcttg aagaccttaa agtaggcgtt 300

caaggtcagc atgcgtcaaa tcttggtgga attattgcaa atgaaatagc aaaagaaata 360

aatgttccag catacatcgt tgatccagtt gttgtggatg agcttgatga agtttcacgt 420

atatcaggaa tggctgacat tccacgtaaa agtatattcc atgcattaaa tcaaaaagca 480

gttgctagac gctatgcaaa agaagttgga aaaaaatacg aagatcttaa tttaatcgtg 540

gtccacatgg gtggcggtac ttcagtaggt actcataaag atggtagagt aattgaagtt 600

aataatacac ttgatggaga aggtccattc tcaccagaaa gaagtggtgg cgttccaata 660

ggcgatcttg tacgtttgtg cttcagcaac aaatatactt atgaagaagt aatgaaaaag 720

ataaacggca aaggcggcgt tgttagttac ttaaatacta tcgattttaa ggctgtagtt 780

gataaagctc ttgaaggcga taagaaatgt gcacttatat atgaagcttt cacattccag 840

gtagcaaaag agataggaaa atgttcaacc gttttaaaag gaaatgtaga tgcaataatc 900

ttaacaggcg gaattgcgta caacgagcat gtatgtaatg ccatagagga tagagtaaaa 960

ttcattgcac ctgtagttcg ttatggtgga gaagatgaac ttcttgcact tgcagaaggt 1020

ggactgcgcg ttttacgcgg agaagaaaaa gctaaggaat acaaataa 1068

<210> SEQ ID NO 65
<211> LENGTH: 1068
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 65

atgtatcgtt tactgattat caatcctggc tcgacctcaa ctaaaattgg tatttatgac 60

gatgaaaaag agatatttga gaagacgtta cgtcattcag ctgaagagat tgaaaaatat 120

aacactatat ttgatcaatt tcagttccgc aagaatgtga ttctcgatgc gttaaaagaa 180

gcaaacattg aagtcagttc tttaaatgct gtagttggac gcggcggact gttaaagcca 240

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attgtcagtg gaacttatgc agtaaatcaa aaaatgcttg aagaccttaa agtgggcgtt 300

caaggtcagc atgccagcaa tcttggtggc attattgcca atgaaatcgc aaaagaaatc 360

aatgttccag catacatcgt tgatccggtt gttgtggatg agcttgatga agttagccgt 420

ataagcggaa tggctgacat tccacgtaaa agtatattcc atgcattaaa tcaaaaagca 480

gttgctcgtc gctatgcaaa agaagttggt aaaaaatacg aagatcttaa tttaatcgtg 540

gtccacatgg gtggcggtac ttcagtaggt actcataaag atggtcgcgt gattgaagtt 600

aataatacac ttgatggcga aggtccattc tcaccagaac gtagtggtgg cgttccaatt 660

ggcgatctgg tacgtttgtg cttcagcaac aaatatactt atgaagaagt gatgaaaaag 720

ataaacggca aaggcggcgt tgttagttac ctgaatacta tcgattttaa ggctgtagtt 780

gataaagcgc ttgaaggcga taagaaatgt gcactgattt atgaagcttt caccttccag 840

gtagcaaaag agattggtaa atgttcaacc gttttaaaag gaaatgttga tgccattatc 900

ttaacaggcg gcattgctta caacgagcat gtatgtaatg ccattgagga tcgcgtaaaa 960

ttcattgcac ctgtagttcg ttatggtggc gaagatgaac tgctggcact ggcagaaggt 1020

ggactgcgcg ttttacgcgg cgaagaaaaa gcgaaggaat acaaataa 1068

<210> SEQ ID NO 66
<211> LENGTH: 1068
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 66

atgtatcgtc tgctgattat caatcctggc tcgacctcaa ctaaaattgg tatttatgac 60

gatgaaaaag agatatttga gaaaacgtta cgtcatagcg ctgaagagat tgaaaaatat 120

aacactattt ttgatcaatt tcagttccgc aagaatgtga ttctcgatgc gctgaaagaa 180

gcaaacattg aagtcagttc gctgaatgcg gtagttggtc gcggcggtct gctgaagcca 240

attgtcagcg gcacttatgc ggtaaatcaa aaaatgctgg aagacctgaa agtgggcgtt 300

caggggcagc atgccagcaa tcttggtggc attattgcca atgaaatcgc caaagaaatc 360

aatgttccgg catacatcgt tgatccggtt gttgtggatg agctggatga agttagccgt 420

atcagcggaa tggctgacat tccacgtaaa agtattttcc atgcactgaa tcaaaaagcg 480

gttgcgcgtc gctatgcaaa agaagttggt aaaaaatacg aagatcttaa tctgatcgtg 540

gtgcatatgg gtggcggtac tagcgtcggt actcataaag atggtcgcgt gattgaagtt 600

aataatacac ttgatggcga aggtccattc tcaccagaac gtagcggtgg cgttccaatt 660

ggcgatctgg tacgtttgtg cttcagcaac aaatatacct atgaagaagt gatgaaaaag 720

ataaacggca aaggcggcgt tgttagttac ctgaatacta tcgattttaa ggcggtagtt 780

gataaagcgc tggaaggcga taagaaatgt gcactgattt atgaagcgtt caccttccag 840

gtggcaaaag agattggtaa atgttcaacc gttctgaaag gcaatgttga tgccattatc 900

ctgaccggcg gcattgctta caacgagcat gtttgtaatg ccattgagga tcgcgtaaaa 960

ttcattgcac ctgtggttcg ttatggtggc gaagatgaac tgctggcact ggcagaaggt 1020

ggtctgcgcg ttttacgcgg cgaagaaaaa gcgaaagaat acaaataa 1068

<210> SEQ ID NO 67
<211> LENGTH: 1068
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 67

atgtatcgtc tgctgattat caacccgggc agcacctcaa ccaaaattgg tatttacgac 60

gatgaaaaag agatttttga aaaaacgctg cgtcacagcg cagaagagat tgaaaaatac 120

aacaccattt tcgatcagtt ccagttccgc aaaaacgtga ttctcgatgc gctgaaagaa 180

gccaatattg aagtctcctc gctgaatgcg gtggtcggtc gcggcggtct gctgaaaccg 240

attgtcagcg gcacttatgc ggttaatcag aaaatgctgg aagatctgaa agtgggcgtg 300

caggggcagc atgccagcaa tctcggcggc attatcgcca atgaaatcgc caaagagatc 360

aacgtgccgg cttatatcgt cgatccggtg gtggttgatg aactggatga agtcagccgt 420

atcagcggca tggcggatat tccgcgtaaa agcattttcc atgcgctgaa tcagaaagcg 480

gttgcgcgtc gctatgccaa agaagtgggt aaaaaatatg aagatctcaa tctgattgtg 540

gtgcatatgg gcggcggcac cagcgtcggt acgcataaag atggtcgcgt gattgaagtg 600

aataacacgc tggatggcga agggccgttc tcgccggaac gtagcggcgg cgtgccgatt 660

ggcgatctgg tgcgtctgtg tttcagcaat aaatacacct acgaagaagt gatgaaaaaa 720

atcaacggca aaggcggcgt ggttagctat ctgaatacca tcgattttaa agcggtggtt 780

gataaagcgc tggaaggcga taaaaaatgc gcgctgattt atgaagcgtt taccttccag 840

gtggcgaaag agattggtaa atgttcaacc gtgctgaaag gcaacgttga tgccattatt 900

ctgaccggcg gcattgctta taacgaacat gtttgtaatg ccattgaaga tcgcgtgaaa 960

tttattgcgc cggtggtgcg ttacggcggc gaagatgaac tgctggcgct ggcggaaggc 1020

ggtctgcgcg tgctgcgcgg cgaagaaaaa gcgaaagagt acaaataa 1068

<210> SEQ ID NO 68
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Clostridium biejerinckii

<400> SEQUENCE: 68

atgaataaag acacactaat acctacaact aaagatttaa aagtaaaaac aaatggtgaa 60

aacattaatt taaagaacta caaggataat tcttcatgtt tcggagtatt cgaaaatgtt 120

gaaaatgcta taagcagcgc tgtacacgca caaaagatat tatcccttca ttatacaaaa 180

gagcaaagag aaaaaatcat aactgagata agaaaggccg cattacaaaa taaagaggtc 240

ttggctacaa tgattctaga agaaacacat atgggaagat atgaggataa aatattaaaa 300

catgaattgg tagctaaata tactcctggt acagaagatt taactactac tgcttggtca 360

ggtgataatg gtcttacagt tgtagaaatg tctccatatg gtgttatagg tgcaataact 420

ccttctacga atccaactga aactgtaata tgtaatagca taggcatgat agctgctgga 480

aatgctgtag tatttaacgg acacccatgc gctaaaaaat gtgttgcctt tgctgttgaa 540

atgataaata aggcaattat ttcatgtggc ggtcctgaaa atctagtaac aactataaaa 600

aatccaacta tggagtctct agatgcaatt attaagcatc cttcaataaa acttctttgc 660

ggaactgggg gtccaggaat ggtaaaaacc ctcttaaatt ctggtaagaa agctataggt 720

gctggtgctg gaaatccacc agttattgta gatgatactg ctgatataga aaaggctggt 780

aggagcatca ttgaaggctg ttcttttgat aataatttac cttgtattgc agaaaaagaa 840

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gtatttgttt ttgagaatgt tgcagatgat ttaatatcta acatgctaaa aaataatgct 900

gtaattataa atgaagatca agtatcaaaa ttaatagatt tagtattaca aaaaaataat 960

gaaactcaag aatactttat aaacaaaaaa tgggtaggaa aagatgcaaa attattctta 1020

gatgaaatag atgttgagtc tccttcaaat gttaaatgca taatctgcga agtaaatgca 1080

aatcatccat ttgttatgac agaactcatg atgccaatat tgccaattgt aagagttaaa 1140

gatatagatg aagctattaa atatgcaaag atagcagaac aaaatagaaa acatagtgcc 1200

tatatttatt ctaaaaatat agacaaccta aatagatttg aaagagaaat agatactact 1260

atttttgtaa agaatgctaa atcttttgct ggtgttggtt atgaagcaga aggatttaca 1320

actttcacta ttgctggatc tactggtgag ggaataacct ctgcaaggaa ttttacaaga 1380

caaagaagat gtgtacttgc cggctaa 1407

<210> SEQ ID NO 69
<211> LENGTH: 468
<212> TYPE: PRT
<213> ORGANISM: Clostridium biejerinckii

<400> SEQUENCE: 69

Met Asn Lys Asp Thr Leu Ile Pro Thr Thr Lys Asp Leu Lys Val Lys
1 5 10 15

Thr Asn Gly Glu Asn Ile Asn Leu Lys Asn Tyr Lys Asp Asn Ser Ser
20 25 30

Cys Phe Gly Val Phe Glu Asn Val Glu Asn Ala Ile Ser Ser Ala Val
35 40 45

His Ala Gln Lys Ile Leu Ser Leu His Tyr Thr Lys Glu Gln Arg Glu
50 55 60

Lys Ile Ile Thr Glu Ile Arg Lys Ala Ala Leu Gln Asn Lys Glu Val
65 70 75 80

Leu Ala Thr Met Ile Leu Glu Glu Thr His Met Gly Arg Tyr Glu Asp
85 90 95

Lys Ile Leu Lys His Glu Leu Val Ala Lys Tyr Thr Pro Gly Thr Glu
100 105 110

Asp Leu Thr Thr Thr Ala Trp Ser Gly Asp Asn Gly Leu Thr Val Val
115 120 125

Glu Met Ser Pro Tyr Gly Val Ile Gly Ala Ile Thr Pro Ser Thr Asn
130 135 140

Pro Thr Glu Thr Val Ile Cys Asn Ser Ile Gly Met Ile Ala Ala Gly
145 150 155 160

Asn Ala Val Val Phe Asn Gly His Pro Cys Ala Lys Lys Cys Val Ala
165 170 175

Phe Ala Val Glu Met Ile Asn Lys Ala Ile Ile Ser Cys Gly Gly Pro
180 185 190

Glu Asn Leu Val Thr Thr Ile Lys Asn Pro Thr Met Glu Ser Leu Asp
195 200 205

Ala Ile Ile Lys His Pro Ser Ile Lys Leu Leu Cys Gly Thr Gly Gly
210 215 220

Pro Gly Met Val Lys Thr Leu Leu Asn Ser Gly Lys Lys Ala Ile Gly
225 230 235 240

Ala Gly Ala Gly Asn Pro Pro Val Ile Val Asp Asp Thr Ala Asp Ile
245 250 255

Glu Lys Ala Gly Arg Ser Ile Ile Glu Gly Cys Ser Phe Asp Asn Asn
260 265 270

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Leu Pro Cys Ile Ala Glu Lys Glu Val Phe Val Phe Glu Asn Val Ala
        275                 280                 285

Asp Asp Leu Ile Ser Asn Met Leu Lys Asn Asn Ala Val Ile Ile Asn
    290                 295                 300

Glu Asp Gln Val Ser Lys Leu Ile Asp Leu Val Leu Gln Lys Asn Asn
305                 310                 315                 320

Glu Thr Gln Glu Tyr Phe Ile Asn Lys Lys Trp Val Gly Lys Asp Ala
                325                 330                 335

Lys Leu Phe Leu Asp Glu Ile Asp Val Glu Ser Pro Ser Asn Val Lys
            340                 345                 350

Cys Ile Ile Cys Glu Val Asn Ala Asn His Pro Phe Val Met Thr Glu
        355                 360                 365

Leu Met Met Pro Ile Leu Pro Ile Val Arg Val Lys Asp Ile Asp Glu
    370                 375                 380

Ala Ile Lys Tyr Ala Lys Ile Ala Glu Gln Asn Arg Lys His Ser Ala
385                 390                 395                 400

Tyr Ile Tyr Ser Lys Asn Ile Asp Asn Leu Asn Arg Phe Glu Arg Glu
                405                 410                 415

Ile Asp Thr Thr Ile Phe Val Lys Asn Ala Lys Ser Phe Ala Gly Val
            420                 425                 430

Gly Tyr Glu Ala Glu Gly Phe Thr Thr Phe Thr Ile Ala Gly Ser Thr
        435                 440                 445

Gly Glu Gly Ile Thr Ser Ala Arg Asn Phe Thr Arg Gln Arg Arg Cys
    450                 455                 460

Val Leu Ala Gly
465
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<210> SEQ ID NO 70
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 70

```
atgaataaag acacactaat acctacaact aaagatttaa aagtaaaaac aaatggtgaa     60

aacattaatt taaagaacta caaggataat tcttcatgtt tcggcgtatt cgaaaatgtt    120

gaaaatgcta taagcagcgc tgtacacgca caaaagatat tatcccttca ttatacaaaa    180

gagcaacgtg aaaaaatcat aactgagata agaaaggccg cattacaaaa taaagaggtc    240

ttggctacaa tgattctgga agaaacacat atgggacgtt atgaggataa aatattaaaa    300

catgaattgg tagctaaata tactcctggt acagaagatt taactactac tgcctggtca    360

ggtgataatg gtctgacagt tgtagaaatg tctccatatg gtgttattgg tgcaataact    420

ccttctacga atccaactga aactgtaata tgtaatagca taggcatgat tgctgctgga    480

aatgctgtag tatttaacgg acacccatgc gctaaaaaat gtgttgcctt tgctgttgaa    540

atgataaata aggcaattat ttcatgtggc ggtcctgaaa atctggtaac aactataaaa    600

aatccaacca tggagtctct ggatgcaatt attaagcatc cttcaataaa acttctttgc    660

ggaactgggg gtccaggaat ggtaaaaacc ctgttaaatt ctggtaagaa agctataggt    720

gctggtgctg gaaatccacc agttattgtc gatgatactg ctgatataga aaaggctggt    780

cgtagcatca ttgaaggctg ttcttttgat aataatttac cttgtattgc agaaaaagaa    840

gtatttgttt ttgagaatgt tgcagatgat ttaatatcta acatgctaaa aaataatgct    900
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gtaattataa atgaagatca agtatcaaaa ttaatcgatt tagtattaca aaaaaataat 960

gaaactcaag aatactttat aaacaaaaaa tgggtaggaa aagatgcaaa attattcctc 1020

gatgaaatag atgttgagtc tccttcaaat gttaaatgca taatctgcga agtaaatgca 1080

aatcatccat ttgttatgac agaactgatg atgccaatat tgccaattgt acgcgttaaa 1140

gatatcgatg aagctattaa atatgcaaag atagcagaac aaaatagaaa acatagtgcc 1200

tatatttatt ctaaaaatat cgacaacctg aatcgctttg aacgtgaaat agatactact 1260

atttttgtaa agaatgctaa atcttttgct ggtgttggtt atgaagcaga aggatttaca 1320

actttcacta ttgctggatc tactggtgag ggaataacct ctgcacgtaa ttttacacgc 1380

caacgtcgct gtgtacttgc cggctaa 1407

<210> SEQ ID NO 71
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 71

atgaataaag acacactgat ccctacaact aaagatttaa aagtaaaaac aaatggtgaa 60

aacattaatt taaagaacta caaagataat agcagttgtt tcggcgtatt cgaaaatgtt 120

gaaaatgcta tcagcagcgc tgtacacgca caaaagatat tatcgctgca ttatacaaaa 180

gagcaacgtg aaaaaatcat cactgagata cgtaaggccg cattacaaaa taaagaggtg 240

ctggctacaa tgattctgga agaaacacat atgggacgtt atgaggataa aatattaaaa 300

catgaactgg tagctaaata tactcctggt acagaagatt taactactac tgcctggagc 360

ggtgataatg gtctgacagt tgtagaaatg tctccatatg gtgttattgg tgcaataact 420

ccttctacca atccaactga aactgtaatt tgtaatagca ttggcatgat tgctgctgga 480

aatgctgtag tatttaacgg acacccatgc gctaaaaaat gtgttgcctt tgctgttgaa 540

atgatcaata aggcaattat tagctgtggc ggtccggaaa atctggtaac aactataaaa 600

aatccaacca tggagtctct ggatgccatt attaagcatc cttcaataaa actgctttgc 660

ggaactggcg gtccaggaat ggtaaaaacc ctgttaaatt ctggtaagaa agctattggt 720

gctggtgctg gaaatccacc agttattgtc gatgatactg ctgatattga aaaggctggt 780

cgtagcatca ttgaaggctg ttcttttgat aataatttac cttgtattgc agaaaaagaa 840

gtatttgttt ttgagaatgt tgcagatgat ttaatatcta acatgctgaa aaataatgct 900

gtaattatca atgaagatca ggtatcaaaa ttaatcgatt tagtattaca aaaaaataat 960

gaaactcaag aatactttat caacaaaaaa tgggtaggta aagatgcaaa attattcctc 1020

gatgaaatcg atgttgagtc tccttcaaat gttaaatgca ttatctgcga agtgaatgcc 1080

aatcatccat ttgttatgac agaactgatg atgccaatat tgccaattgt gcgcgttaaa 1140

gatatcgatg aagctattaa atatgcaaag attgcagaac aaaatagaaa acatagtgcc 1200

tatatttata gcaaaaatat cgacaacctg aatcgctttg aacgtgaaat cgatactact 1260

atttttgtaa agaatgctaa atcttttgct ggtgttggtt atgaagcaga aggatttacc 1320

actttcacta ttgctggatc tactggtgag ggcataacct ctgcacgtaa ttttacccgc 1380

caacgtcgct gtgtactggc cggctaa 1407

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<210> SEQ ID NO 72
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 72

atgaataaag acacgctgat cccgacaact aaagatctga aagtaaaaac caatggtgaa 60

aacattaatc tgaagaacta caaagataat agcagttgtt tcggcgtatt cgaaaatgtt 120

gaaaatgcta tcagcagcgc ggtacacgca caaaagatac tctcgctgca ttataccaaa 180

gagcaacgtg aaaaaatcat cactgagatc cgtaaggccg cattacaaaa taaagaggtg 240

ctggcaacaa tgattctgga agaaacacat atgggacgtt atgaggataa aatactgaaa 300

catgaactgg tggcgaaata tacgcctggt actgaagatt taaccaccac tgcctggagc 360

ggtgataatg gtctgaccgt tgtggaaatg tcgccttatg gtgttattgg tgcaattacg 420

ccttcaacca atccaactga aacggtaatt tgtaatagca ttggcatgat tgctgctgga 480

aatgcggtag tatttaacgg tcacccctgc gctaaaaaat gtgttgcctt tgctgttgaa 540

atgatcaata aagcgattat tagctgtggc ggtccggaaa atctggtaac cactataaaa 600

aatccaacca tggagtcgct ggatgccatt attaagcatc cttcaatcaa actgctgtgc 660

ggcactggcg gtccaggaat ggtgaaaacc ctgctgaata gcggtaagaa agcgattggt 720

gctggtgctg gaaatccacc agttattgtc gatgatactg ctgatattga aaaagcgggt 780

cgtagcatca ttgaaggctg ttcttttgat aataatttac cttgtattgc agaaaaagaa 840

gtatttgttt ttgagaatgt tgccgatgat ctgatctcta acatgctgaa aaataatgcg 900

gtgattatca atgaagatca ggttagcaaa ctgatcgatc tggtattaca aaaaaataat 960

gaaactcaag aatactttat caacaaaaaa tgggtaggta aagatgcaaa actgttcctc 1020

gatgaaatcg atgttgagtc gccttcaaat gttaaatgca ttatctgcga agtgaatgcc 1080

aatcatccat ttgtgatgac cgaactgatg atgccaattt tgccgattgt gcgcgttaaa 1140

gatatcgatg aagcgattaa atatgcaaag attgcagaac aaaatcgtaa acatagtgcc 1200

tatatttata gcaaaaatat cgacaacctg aatcgctttg aacgtgaaat cgataccact 1260

atttttgtga agaatgctaa atcttttgct ggtgttggtt atgaagcaga aggttttacc 1320

actttcacta ttgctggaag caccggtgaa ggcattacct ctgcacgtaa ttttacccgc 1380

caacgtcgct gtgtactggc cggctaa 1407

<210> SEQ ID NO 73
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 73

atgaataaag atacgctgat cccgaccacc aaagatctga aagtgaaaac caacggcgaa 60

aatatcaacc tgaaaaacta taaagataac agcagttgct ttggcgtgtt tgaaaacgtt 120

gaaaacgcca tctccagcgc ggtgcatgcg caaaaaattc tctcgctgca ttacaccaaa 180

gagcagcgtg aaaaaattat caccgaaatc cgtaaagcgg cgctgcaaaa caaagaagtg 240

ctggcaacca tgatcctgga agaaacgcat atggggcgtt atgaagataa aattctgaaa 300

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catgaactgg tggcgaaata cacgccgggc actgaagatc tgaccaccac cgcctggagc 360

ggcgataacg gcctgaccgt ggtggagatg tcgccttatg gcgtgattgg cgcgattacg 420

ccgtcaacca acccgaccga aacggtgatt tgtaacagca ttggcatgat tgccgcgggt 480

aatgcggtgg tgtttaacgg tcatccctgc gcgaaaaaat gtgtggcgtt tgccgttgag 540

atgatcaaca aagcgattat cagctgcggc ggcccggaaa atctggtgac caccatcaaa 600

aatccgacca tggaatcgct ggatgccatt atcaaacatc cttccatcaa actgctgtgc 660

ggcaccggcg gcccgggcat ggtgaaaacg ctgctgaaca gcggtaaaaa agcgattggc 720

gcgggcgcgg gtaacccgcc ggtgattgtc gatgacaccg ccgatattga aaaagcgggg 780

cgtagcatta ttgaaggctg ttcttttgat aacaacctgc cctgcattgc cgaaaaagaa 840

gtgtttgtct ttgaaaacgt cgccgatgat ctgatcagca atatgctgaa aaacaacgcg 900

gtgattatca atgaagatca ggttagcaaa ctgatcgatc tggtgctgca aaaaaacaac 960

gaaacgcagg aatattttat caacaaaaaa tgggttggta aagatgccaa actgtttctc 1020

gatgaaatcg atgttgaatc gccgtctaac gtgaaatgta ttatctgcga agtgaacgcc 1080

aaccatccgt ttgtgatgac cgaactgatg atgccgattc tgccgattgt gcgcgtgaaa 1140

gatatcgatg aagcgattaa atatgccaaa attgccgaac aaaaccgtaa acacagcgcc 1200

tatatttaca gcaaaaatat cgataacctg aaccgctttg aacgtgaaat cgataccacc 1260

atttttgtga aaaatgccaa aagttttgcc ggcgttggtt atgaagcgga aggttttacc 1320

acctttacca ttgccggtag caccggcgaa ggcattacca gcgcccgtaa ttttacccgc 1380

cagcgtcgct gcgtgctggc gggctaa 1407

<210> SEQ ID NO 74
<211> LENGTH: 1023
<212> TYPE: DNA
<213> ORGANISM: Geobacillus thermoglucosidasius

<400> SEQUENCE: 74

atgaaagctg cagtagtaga gcaatttaag gaaccattaa aaattaaaga agtggaaaag 60

ccatctattt catatggcga agtattagtc cgcattaaag catgcggtgt atgccatacg 120

gacttgcacg ccgctcatgg cgattggcca gtaaaaccaa aacttccttt aatccctggc 180

catgaaggag tcggaattgt tgaagaagtc ggtccggggg taacccattt aaaagtggga 240

gaccgcgttg gaattccttg gttatattct gcgtgcggcc attgcgaata ttgtttaagc 300

ggacaagaag cattatgtga acatcaacaa aacgccggct actcagtcga cgggggttat 360

gcagaatatt gcagagctgc gccagattat gtggtgaaaa ttcctgacaa cttatcgttt 420

gaagaagctg ctcctatttt ctgcgccgga gttactactt ataaagcgtt aaaagtcaca 480

ggtacaaaac cgggagaatg ggtagcgatc tatggcatcg gcggccttgg acatgttgcc 540

gtccagtatg cgaaagcgat ggggcttcat gttgttgcag tggatatcgg cgatgagaaa 600

ctggaacttg caaaagagct tggcgccgat cttgttgtaa atcctgcaaa agaaaatgcg 660

gcccaattta tgaaagagaa agtcggcgga gtacacgcgg ctgttgtgac agctgtatct 720

aaacctgctt ttcaatctgc gtacaattct atccgcagag gcggcacgtg cgtgcttgtc 780

ggattaccgc cggaagaaat gcctattcca atctttgata cggtattaaa cggaattaaa 840

attatcggtt ccattgtcgg cacgcggaaa gacttgcaag aagcgcttca gttcgctgca 900

gaaggtaaag taaaaaccat tattgaagtg caacctcttg aaaaaattaa cgaagtattt 960

gacagaatgc taaaaggaga aattaacgga cgggttgttt taacgttaga aaataataat 1020

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taa 1023

<210> SEQ ID NO 75
<211> LENGTH: 340
<212> TYPE: PRT
<213> ORGANISM: Geobacillus thermoglucosidasius

<400> SEQUENCE: 75

Met Lys Ala Ala Val Val Glu Gln Phe Lys Glu Pro Leu Lys Ile Lys
1 5 10 15

Glu Val Glu Lys Pro Ser Ile Ser Tyr Gly Glu Val Leu Val Arg Ile
20 25 30

Lys Ala Cys Gly Val Cys His Thr Asp Leu His Ala Ala His Gly Asp
35 40 45

Trp Pro Val Lys Pro Lys Leu Pro Leu Ile Pro Gly His Glu Gly Val
50 55 60

Gly Ile Val Glu Glu Val Gly Pro Gly Val Thr His Leu Lys Val Gly
65 70 75 80

Asp Arg Val Gly Ile Pro Trp Leu Tyr Ser Ala Cys Gly His Cys Glu
85 90 95

Tyr Cys Leu Ser Gly Gln Glu Ala Leu Cys Glu His Gln Gln Asn Ala
100 105 110

Gly Tyr Ser Val Asp Gly Gly Tyr Ala Glu Tyr Cys Arg Ala Ala Pro
115 120 125

Asp Tyr Val Val Lys Ile Pro Asp Asn Leu Ser Phe Glu Glu Ala Ala
130 135 140

Pro Ile Phe Cys Ala Gly Val Thr Thr Tyr Lys Ala Leu Lys Val Thr
145 150 155 160

Gly Thr Lys Pro Gly Glu Trp Val Ala Ile Tyr Gly Ile Gly Gly Leu
165 170 175

Gly His Val Ala Val Gln Tyr Ala Lys Ala Met Gly Leu His Val Val
180 185 190

Ala Val Asp Ile Gly Asp Glu Lys Leu Glu Leu Ala Lys Glu Leu Gly
195 200 205

Ala Asp Leu Val Val Asn Pro Ala Lys Glu Asn Ala Ala Gln Phe Met
210 215 220

Lys Glu Lys Val Gly Gly Val His Ala Ala Val Val Thr Ala Val Ser
225 230 235 240

Lys Pro Ala Phe Gln Ser Ala Tyr Asn Ser Ile Arg Arg Gly Gly Thr
245 250 255

Cys Val Leu Val Gly Leu Pro Pro Glu Glu Met Pro Ile Pro Ile Phe
260 265 270

Asp Thr Val Leu Asn Gly Ile Lys Ile Ile Gly Ser Ile Val Gly Thr
275 280 285

Arg Lys Asp Leu Gln Glu Ala Leu Gln Phe Ala Ala Glu Gly Lys Val
290 295 300

Lys Thr Ile Ile Glu Val Gln Pro Leu Glu Lys Ile Asn Glu Val Phe
305 310 315 320

Asp Arg Met Leu Lys Gly Glu Ile Asn Gly Arg Val Val Leu Thr Leu
325 330 335

Glu Asn Asn Asn
340

<210> SEQ ID NO 76

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<211> LENGTH: 4090
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 76

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ctggtcaaag tgggcgacaa agttgaagcc gaacagtcgc tgatcaccgt agaaggcgac 120

aaagcctcta tggaagttcc gtctccgcag gcgggtatcg ttaaagagat caaagtctct 180

gttggcgata aaacccagac cggcgcactg attatgattt tcgattccgc cgacggtgca 240

gcagacgctg cacctgctca ggcagaagag aagaaagaag cagctccggc agcagcacca 300

gcggctgcgg cggcaaaaga cgttaacgtt ccggatatcg gcagcgacga agttgaagtg 360

accgaaatcc tggtgaaagt tggcgataaa gttgaagctg aacagtcgct gatcaccgta 420

gaaggcgaca aggcttctat ggaagttccg gctccgtttg ctggcaccgt gaaagagatc 480

aaagtgaacg tgggtgacaa agtgtctacc ggctcgctga ttatggtctt cgaagtcgcg 540

ggtgaagcag gcgcggcagc tccggccgct aaacaggaag cagctccggc agcggcccct 600

gcaccagcgg ctggcgtgaa agaagttaac gttccggata tcggcggtga cgaagttgaa 660

gtgactgaag tgatggtgaa agtgggcgac aaagttgccg ctgaacagtc actgatcacc 720

gtagaaggcg acaaagcttc tatggaagtt ccggcgccgt ttgcaggcgt cgtgaaggaa 780

ctgaaagtca acgttggcga taaagtgaaa actggctcgc tgattatgat cttcgaagtt 840

gaaggcgcag cgcctgcggc agctcctgcg aaacaggaag cggcagcgcc ggcaccggca 900

gcaaaagctg aagccccggc agcagcacca gctgcgaaag cggaaggcaa atctgaattt 960

gctgaaaacg acgcttatgt tcacgcgact ccgctgatcc gccgtctggc acgcgagttt 1020

ggtgttaacc ttgcgaaagt gaagggcact ggccgtaaag gtcgtatcct gcgcgaagac 1080

gttcaggctt acgtgaaaga agctatcaaa cgtgcagaag cagctccggc agcgactggc 1140

ggtggtatcc ctggcatgct gccgtggccg aaggtggact tcagcaagtt tggtgaaatc 1200

gaagaagtgg aactgggccg catccagaaa atctctggtg cgaacctgag ccgtaactgg 1260

gtaatgatcc cgcatgttac tcacttcgac aaaaccgata tcaccgagtt ggaagcgttc 1320

cgtaaacagc agaacgaaga agcggcgaaa cgtaagctgg atgtgaagat caccccggtt 1380

gtcttcatca tgaaagccgt tgctgcagct cttgagcaga tgcctcgctt caatagttcg 1440

ctgtcggaag acggtcagcg tctgaccctg aagaaataca tcaacatcgg tgtggcggtg 1500

gataccccga acggtctggt tgttccggta ttcaaagacg tcaacaagaa aggcatcatc 1560

gagctgtctc gcgagctgat gactatttct aagaaagcgc gtgacggtaa gctgactgcg 1620

ggcgaaatgc agggcggttg cttcaccatc tccagcatcg gcggcctggg tactacccac 1680

ttcgcgccga ttgtgaacgc gccggaagtg gctatcctcg gcgtttccaa gtccgcgatg 1740

gagccggtgt ggaatggtaa agagttcgtg ccgcgtctga tgctgccgat ttctctctcc 1800

ttcgaccacc gcgtgatcga cggtgctgat ggtgcccgtt tcattaccat cattaacaac 1860

acgctgtctg acattcgccg tctggtgatg taagtaaaag agccggccca acggccggct 1920

tttttctggt aatctcatga atgtattgag gttattagcg aatagacaaa tcggttgccg 1980

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gtggttaggg tattacttca cataccctat ggatttctgg gtgcagcaag gtagcaagcg 2100

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ccagaatccc caggagctta cataagtaag tgactggggt gagggcgtga agctaacgcc 2160

gctgcggcct gaaagacgac gggtatgacc gccggagata aatatataga ggtcatgatg 2220

agtactgaaa tcaaaactca ggtcgtggta cttggggcag gccccgcagg ttactccgct 2280

gccttccgtt gcgctgattt aggtctggaa accgtaatcg tagaacgtta caacaccctt 2340

ggcggtgttt gtctgaacgt gggttgtatc ccttctaaag cgctgctgca cgtggcaaaa 2400

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gacattgaca agatccgcac ctggaaagaa aaagtcatca ctcagctgac cggtggtctg 2520

gctggcatgg ccaaaggtcg taaagtgaag gtggttaacg gtctgggtaa atttaccggc 2580

gctaacaccc tggaagtgga aggcgaaaac ggcaaaaccg tgatcaactt cgacaacgcc 2640

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gacgtggtgg aaatgttcga ccaggttatc ccggctgccg acaaagacgt ggtgaaagtc 2880

ttcaccaaac gcatcagcaa gaaatttaac ctgatgctgg aagccaaagt gactgccgtt 2940

gaagcgaaag aagacggtat ttacgtttcc atggaaggta aaaaagcacc ggcggaagcg 3000

cagcgttacg acgcagtgct ggtcgctatc ggccgcgtac cgaatggtaa aaacctcgat 3060

gcaggtaaag ctggcgtgga agttgacgat cgcggcttca tccgcgttga caaacaaatg 3120

cgcaccaacg tgccgcacat ctttgctatc ggcgatatcg tcggtcagcc gatgctggcg 3180

cacaaaggtg tccatgaagg ccacgttgcc gcagaagtta tctccggtct gaaacactac 3240

ttcgatccga aagtgatccc atccatcgcc tacactaaac cagaagtggc atgggtcggt 3300

ctgaccgaga aagaagcgaa agagaaaggc atcagctacg aaaccgccac cttcccgtgg 3360

gctgcttccg gccgtgctat cgcttctgac tgcgcagatg gtatgaccaa actgatcttc 3420

gacaaagaga cccaccgtgt tatcggcggc gcgattgtcg gcaccaacgg cggcgagctg 3480

ctgggtgaga tcggcctggc tatcgagatg ggctgtgacg ctgaagacat cgccctgacc 3540

atccacgctc acccgactct gcacgagtcc gttggcctgg cggcggaagt gttcgaaggc 3600

agcatcaccg acctgccaaa cgccaaagcg aagaaaaagt aacttttttct ttcaggaaaa 3660

aagcataagc ggctccggga gccgcttttt ttatgcctga tgtttagaac tatgtcactg 3720

ttcataaacc gctacacctc atacatactt taagggcgaa ttctgcagat atccatcaca 3780

ctggcggccg ctcgagcatg catctagcac atccggcaat taaaaaagcg gctaaccacg 3840

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gttcgcatac tgctgaccgt tgctcgttat tcagcctgac agtatggtta ctgtcgttta 3960

gacgttgtgg gcggctctcc tgaactttct cccgaaaaac ctgacgttgt tcaggtgatg 4020

ccgattgaac acgctggcgg gcgttatcac gttgctgttg attcagtggg cgctgctgta 4080

ctttttcctt 4090

<210> SEQ ID NO 77
<211> LENGTH: 475
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 77

Met Met Ser Thr Glu Ile Lys Thr Gln Val Val Val Leu Gly Ala Gly
1 5 10 15

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Pro Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu
            20                  25                  30

Thr Val Ile Val Glu Arg Tyr Asn Thr Leu Gly Gly Val Cys Leu Asn
        35                  40                  45

Val Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys Val Ile
    50                  55                  60

Glu Glu Ala Lys Ala Leu Ala Glu His Gly Ile Val Phe Gly Glu Pro
65                  70                  75                  80

Lys Thr Asp Ile Asp Lys Ile Arg Thr Trp Lys Glu Lys Val Ile Asn
                85                  90                  95

Gln Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Gly Arg Lys Val Lys
            100                 105                 110

Val Val Asn Gly Leu Gly Lys Phe Thr Gly Ala Asn Thr Leu Glu Val
        115                 120                 125

Glu Gly Glu Asn Gly Lys Thr Val Ile Asn Phe Asp Asn Ala Ile Ile
    130                 135                 140

Ala Ala Gly Ser Arg Pro Ile Gln Leu Pro Phe Ile Pro His Glu Asp
145                 150                 155                 160

Pro Arg Ile Trp Asp Ser Thr Asp Ala Leu Glu Leu Lys Glu Val Pro
                165                 170                 175

Glu Arg Leu Leu Val Met Gly Gly Gly Ile Ile Gly Leu Glu Met Gly
            180                 185                 190

Thr Val Tyr His Ala Leu Gly Ser Gln Ile Asp Val Val Glu Met Phe
        195                 200                 205

Asp Gln Val Ile Pro Ala Ala Asp Lys Asp Ile Val Lys Val Phe Thr
    210                 215                 220

Lys Arg Ile Ser Lys Lys Phe Asn Leu Met Leu Glu Thr Lys Val Thr
225                 230                 235                 240

Ala Val Glu Ala Lys Glu Asp Gly Ile Tyr Val Thr Met Glu Gly Lys
                245                 250                 255

Lys Ala Pro Ala Glu Pro Gln Arg Tyr Asp Ala Val Leu Val Ala Ile
            260                 265                 270

Gly Arg Val Pro Asn Gly Lys Asn Leu Asp Ala Gly Lys Ala Gly Val
        275                 280                 285

Glu Val Asp Asp Arg Gly Phe Ile Arg Val Asp Lys Gln Leu Arg Thr
    290                 295                 300

Asn Val Pro His Ile Phe Ala Ile Gly Asp Ile Val Gly Gln Pro Met
305                 310                 315                 320

Leu Ala His Lys Gly Val His Glu Gly His Val Ala Ala Glu Val Ile
                325                 330                 335

Ala Gly Lys Lys His Tyr Phe Asp Pro Lys Val Ile Pro Ser Ile Ala
            340                 345                 350

Tyr Thr Glu Pro Glu Val Ala Trp Val Gly Leu Thr Glu Lys Glu Ala
        355                 360                 365

Lys Glu Lys Gly Ile Ser Tyr Glu Thr Ala Thr Phe Pro Trp Ala Ala
    370                 375                 380

Ser Gly Arg Ala Ile Ala Ser Asp Cys Ala Asp Gly Met Thr Lys Leu
385                 390                 395                 400

Ile Phe Asp Lys Glu Ser His Arg Val Ile Gly Gly Ala Ile Val Gly
                405                 410                 415

Thr Asn Gly Gly Glu Leu Leu Gly Glu Ile Gly Leu Ala Ile Glu Met
            420                 425                 430

Gly Cys Asp Ala Glu Asp Ile Ala Leu Thr Ile His Ala His Pro Thr
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        435                 440                 445

Leu His Glu Ser Val Gly Leu Ala Ala Glu Val Phe Glu Gly Ser Ile
    450                 455                 460

Thr Asp Leu Pro Asn Pro Lys Ala Lys Lys Lys
465                 470                 475


<210> SEQ ID NO 78
<211> LENGTH: 475
<212> TYPE: PRT
<213> ORGANISM: Klebsiella pneumoniae

<400> SEQUENCE: 78

Met Met Ser Thr Glu Ile Lys Thr Gln Val Val Val Leu Gly Ala Gly
1               5                   10                  15

Pro Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu
            20                  25                  30

Thr Val Ile Val Glu Arg Tyr Ser Thr Leu Gly Gly Val Cys Leu Asn
        35                  40                  45

Val Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys Val Ile
    50                  55                  60

Glu Glu Ala Lys Ala Leu Ala Glu His Gly Ile Val Phe Gly Glu Pro
65                  70                  75                  80

Lys Thr Asp Ile Asp Lys Ile Arg Thr Trp Lys Glu Lys Val Ile Thr
                85                  90                  95

Gln Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Gly Arg Lys Val Lys
            100                 105                 110

Val Val Asn Gly Leu Gly Lys Phe Thr Gly Ala Asn Thr Leu Glu Val
        115                 120                 125

Glu Gly Glu Asn Gly Lys Thr Val Ile Asn Phe Asp Asn Ala Ile Ile
    130                 135                 140

Ala Ala Gly Ser Arg Pro Ile Gln Leu Pro Phe Ile Pro His Glu Asp
145                 150                 155                 160

Pro Arg Val Trp Asp Ser Thr Asp Ala Leu Glu Leu Lys Ser Val Pro
                165                 170                 175

Lys Arg Met Leu Val Met Gly Gly Gly Ile Ile Gly Leu Glu Met Gly
            180                 185                 190

Thr Val Tyr His Ala Leu Gly Ser Glu Ile Asp Val Val Glu Met Phe
        195                 200                 205

Asp Gln Val Ile Pro Ala Ala Asp Lys Asp Val Val Lys Val Phe Thr
    210                 215                 220

Lys Arg Ile Ser Lys Lys Phe Asn Leu Met Leu Glu Ala Lys Val Thr
225                 230                 235                 240

Ala Val Glu Ala Lys Glu Asp Gly Ile Tyr Val Ser Met Glu Gly Lys
                245                 250                 255

Lys Ala Pro Ala Glu Ala Gln Arg Tyr Asp Ala Val Leu Val Ala Ile
            260                 265                 270

Gly Arg Val Pro Asn Gly Lys Asn Leu Asp Ala Gly Lys Ala Gly Val
        275                 280                 285

Glu Val Asp Asp Arg Gly Phe Ile Arg Val Asp Lys Gln Met Arg Thr
    290                 295                 300

Asn Val Pro His Ile Phe Ala Ile Gly Asp Ile Val Gly Gln Pro Met
305                 310                 315                 320

Leu Ala His Lys Gly Val His Glu Gly His Val Ala Ala Glu Val Ile
                325                 330                 335
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Ser Gly Leu Lys His Tyr Phe Asp Pro Lys Val Ile Pro Ser Ile Ala
            340                 345                 350

Tyr Thr Lys Pro Glu Val Ala Trp Val Gly Leu Thr Glu Lys Glu Ala
        355                 360                 365

Lys Glu Lys Gly Ile Ser Tyr Glu Thr Ala Thr Phe Pro Trp Ala Ala
    370                 375                 380

Ser Gly Arg Ala Ile Ala Ser Asp Cys Ala Asp Gly Met Thr Lys Leu
385                 390                 395                 400

Ile Phe Asp Lys Glu Thr His Arg Val Ile Gly Gly Ala Ile Val Gly
                405                 410                 415

Thr Asn Gly Gly Glu Leu Leu Gly Glu Ile Gly Leu Ala Ile Glu Met
            420                 425                 430

Gly Cys Asp Ala Glu Asp Ile Ala Leu Thr Ile His Ala His Pro Thr
        435                 440                 445

Leu His Glu Ser Val Gly Leu Ala Ala Glu Val Phe Glu Gly Ser Ile
    450                 455                 460

Thr Asp Leu Pro Asn Ala Lys Ala Lys Lys Lys
465                 470                 475


<210> SEQ ID NO 79
<211> LENGTH: 347
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polynucleotide

<400> SEQUENCE: 79

ataataatac atatgaacca tgcgagttac gggcctataa gccaggcgag atatgatcta     60

tatcaatttc tcatctataa tgctttgtta gtatctcgtc gccgacttaa taaagagaga    120

gttagtgtga aagctgacaa ccctttttgat cttttacttc ctgctgcaat ggccaaagtg    180

gccgaagagg cgggtgtcta taaagcaacg aaacatccgc ttaagacttt ctatctggcg    240

attaccgccg gtgttttcat ctcaatcgca ttcaccactg gcacaggcac agaaggtagg    300

tgttacatgt cagaacgttt acacaatgac gtggatccta ttattat                  347


<210> SEQ ID NO 80
<211> LENGTH: 4678
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polynucleotide

<400> SEQUENCE: 80

aagaggtaaa agaataatgg ctatcgaaat caaagtaccg gacatcgggg ctgatgaagt     60

tgaaatcacc gagatcctgg tcaaagtggg cgacaaagtt gaagccgaac agtcgctgat    120

caccgtagaa ggcgacaaag cctctatgga agttccgtct ccgcaggcgg gtatcgttaa    180

agagatcaaa gtctctgttg gcgataaaac ccagaccggc gcactgatta tgattttcga    240

ttccgccgac ggtgcagcag acgctgcacc tgctcaggca gaagagaaga aagaagcagc    300

tccggcagca gcaccagcgg ctgcggcggc aaaagacgtt aacgttccgg atatcggcag    360

cgacgaagtt gaagtgaccg aaatcctggt gaaagttggc gataaagttg aagctgaaca    420

gtcgctgatc accgtagaag gcgacaaggc ttctatggaa gttccggctc cgtttgctgg    480

caccgtgaaa gagatcaaag tgaacgtggg tgacaaagtg tctaccggct cgctgattat    540
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ggtcttcgaa gtcgcgggtg aagcaggcgc ggcagctccg gccgctaaac aggaagcagc 600

tccggcagcg gcccctgcac cagcggctgg cgtgaaagaa gttaacgttc cggatatcgg 660

cggtgacgaa gttgaagtga ctgaagtgat ggtgaaagtg ggcgacaaag ttgccgctga 720

acagtcactg atcaccgtag aaggcgacaa agcttctatg gaagttccgg cgccgtttgc 780

aggcgtcgtg aaggaactga aagtcaacgt tggcgataaa gtgaaaactg gctcgctgat 840

tatgatcttc gaagttgaag gcgcagcgcc tgcggcagct cctgcgaaac aggaagcggc 900

agcgccggca ccggcagcaa aagctgaagc cccggcagca gcaccagctg cgaaagcgga 960

aggcaaatct gaatttgctg aaaacgacgc ttatgttcac gcgactccgc tgatccgccg 1020

tctggcacgc gagtttggtg ttaaccttgc gaaagtgaag ggcactggcc gtaaaggtcg 1080

tatcctgcgc gaagacgttc aggcttacgt gaaagaagct atcaaacgtg cagaagcagc 1140

tccggcagcg actggcggtg gtatccctgg catgctgccg tggccgaagg tggacttcag 1200

caagtttggt gaaatcgaag aagtggaact gggccgcatc cagaaaatct ctggtgcgaa 1260

cctgagccgt aactgggtaa tgatcccgca tgttactcac ttcgacaaaa ccgatatcac 1320

cgagttggaa gcgttccgta aacagcagaa cgaagaagcg gcgaaacgta agctggatgt 1380

gaagatcacc ccggttgtct tcatcatgaa agccgttgct gcagctcttg agcagatgcc 1440

tcgcttcaat agttcgctgt cggaagacgg tcagcgtctg accctgaaga aatacatcaa 1500

catcggtgtg gcggtggata ccccgaacgg tctggttgtt ccggtattca aagacgtcaa 1560

caagaaaggc atcatcgagc tgtctcgcga gctgatgact atttctaaga aagcgcgtga 1620

cggtaagctg actgcgggcg aaatgcaggg cggttgcttc accatctcca gcatcggcgg 1680

cctgggtact acccacttcg cgccgattgt gaacgcgccg gaagtggcta tcctcggcgt 1740

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gccgatttct ctctccttcg accaccgcgt gatcgacggt gctgatggtg cccgtttcat 1860

taccatcatt aacaacacgc tgtctgacat tcgccgtctg gtgatgtaag taaaagagcc 1920

ggcccaacgg ccggcttttt tctggtaatc tcatgaatgt attgaggtta ttagcgaata 1980

gacaaatcgg ttgccgtttg ttaagccagg cgagatatga tctatatcaa tttctcatct 2040

ataatgcttt gttagtatct cgtcgccgac ttaataaaga gagagttagt cttctatatc 2100

acagcaagaa ggtaggtgtt acatgatgag tactgaaatc aaaactcagg tcgtggtact 2160

tggggcaggc cccgcaggtt actctgcagc cttccgttgc gctgatttag gtctggaaac 2220

cgtcatcgta gaacgttaca gcaccctcgg tggtgtttgt ctgaacgtgg gttgtatccc 2280

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cggcatcgtt ttcggcgaac cgaaaactga cattgacaag atccgcacct ggaaagaaaa 2400

agtcatcact cagctgaccg gtggtctggc tggcatggcc aaaggtcgta aagtgaaggt 2460

ggttaacggt ctgggtaaat ttaccggcgc taacaccctg gaagtggaag gcgaaaacgg 2520

caaaaccgtg atcaacttcg acaacgccat catcgcggcg ggttcccgtc cgattcagct 2580

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cgtataccat gcgctgggtt cagagattga cgtggtggaa atgttcgacc aggttatccc 2760

ggctgccgac aaagacgtgg tgaaagtctt caccaaacgc atcagcaaga aatttaacct 2820

gatgctggaa gccaaagtga ctgccgttga agcgaaagaa gacggtattt acgtttccat 2880

ggaaggtaaa aaagcaccgg cggaagcgca gcgttacgac gcagtgctgg tcgctatcgg 2940

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ccgcgtaccg aatggtaaaa acctcgatgc aggtaaagct ggcgtggaag ttgacgatcg 3000

cggcttcatc cgcgttgaca aacaaatgcg caccaacgtg ccgcacatct ttgctatcgg 3060

cgatatcgtc ggtcagccga tgctggcgca caaaggtgtc catgaaggcc acgttgccgc 3120

agaagttatc tccggtctga aacactactt cgatccgaaa gtgatcccat ccatcgccta 3180

cactaaacca gaagtggcat gggtcggtct gaccgagaaa gaagcgaaag agaaaggcat 3240

cagctacgaa accgccacct tcccgtgggc tgcttccggc cgtgctatcg cttctgactg 3300

cgcagatggt atgaccaaac tgatcttcga caaagagacc caccgtgtta tcggcggcgc 3360

gattgtcggc accaacggcg gcgagctgct gggtgagatc ggcctggcta tcgagatggg 3420

ctgtgacgct gaagacatcg ccctgaccat ccacgctcac ccgactctgc acgagtccgt 3480

tggcctggcg gcggaagtgt tcgaaggcag catcaccgac ctgccaaacg ccaaagcgaa 3540

gaaaaagtaa ctttttcttt caggaaaaaa gcataagcgg ctccgggagc cgcttttttt 3600

atgcctgatg tttagaacta tgtcactgtt cataaaccgc tacacctcat acatacttta 3660

agggcgaatt ctgcagatat ccatcacact ggcggccgct cgagcatgca tctagcacat 3720

ccggcaatta aaaaagcggc taaccacgcc gctttttttа cgtctgcaat ttacctttcc 3780

agtcttcttg ctccacgttc agagagacgt tcgcatactg ctgaccgttg ctcgttattc 3840

agcctgacag tatggttact gtcgtttaga cgttgtgggc ggctctcctg aactttctcc 3900

cgaaaaacct gacgttgttc aggtgatgcc gattgaacac gctggcgggc gttatcacgt 3960

tgctgttgat tcagtgggcg ctgctgtact ttttccttaa acacctggcg ctgctctggt 4020

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gctgcctctc ttcgctgctg gatcttcggg ttagtctgca ttctctcgcg aactgcctgg 4200

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tcagtaagat gctcaccggt gatacggttg aaattgttga cgtcgatatt gatgttgtcg 4620

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<210> SEQ ID NO 81
<211> LENGTH: 1114
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (323)..(958)

<400> SEQUENCE: 81

caaaaaaccg gagtctgtgc tccggttttt tattatccgc taatcaatta catatgaata 60

tcctccttag ttcctattcc gaagttccta ttctctagaa agtataggaa cttcggcgcg 120

cctacctgtg acggaagatc acttcgcaga ataaataaat cctggtgtcc ctgttgatac 180

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cgggaagccc tgggccaact tttggcgaaa atgagacgtt gatcggcacg taagaggttc    240

caactttcac cataatgaaa taagatcact accgggcgta ttttttgagt tgtcgagatt    300

ttcaggagct aaggaagcta aa atg gag aaa aaa atc act gga tat acc acc      352
                        Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr
                        1               5                   10

gtt gat ata tcc caa tgg cat cgt aaa gaa cat ttt gag gca ttt cag      400
Val Asp Ile Ser Gln Trp His Arg Lys Glu His Phe Glu Ala Phe Gln
                15                  20                  25

tca gtt gct caa tgt acc tat aac cag acc gtt cag ctg gat att acg      448
Ser Val Ala Gln Cys Thr Tyr Asn Gln Thr Val Gln Leu Asp Ile Thr
            30                  35                  40

gcc ttt tta aag acc gta aag aaa aat aag cac aag ttt tat ccg gcc      496
Ala Phe Leu Lys Thr Val Lys Lys Asn Lys His Lys Phe Tyr Pro Ala
        45                  50                  55

ttt att cac att ctt gcc cgc ctg atg aat gct cat ccg gaa tta cgt      544
Phe Ile His Ile Leu Ala Arg Leu Met Asn Ala His Pro Glu Leu Arg
    60                  65                  70

atg gca atg aaa gac ggt gag ctg gtg ata tgg gat agt gtt cac cct      592
Met Ala Met Lys Asp Gly Glu Leu Val Ile Trp Asp Ser Val His Pro
75                  80                  85                  90

tgt tac acc gtt ttc cat gag caa act gaa acg ttt tca tcg ctc tgg      640
Cys Tyr Thr Val Phe His Glu Gln Thr Glu Thr Phe Ser Ser Leu Trp
                95                  100                 105

agt gaa tac cac gac gat ttc cgg cag ttt cta cac ata tat tcg caa      688
Ser Glu Tyr His Asp Asp Phe Arg Gln Phe Leu His Ile Tyr Ser Gln
            110                 115                 120

gat gtg gcg tgt tac ggt gaa aac ctg gcc tat ttc cct aaa ggg ttt      736
Asp Val Ala Cys Tyr Gly Glu Asn Leu Ala Tyr Phe Pro Lys Gly Phe
        125                 130                 135

att gag aat atg ttt ttc gtc tca gcc aat ccc tgg gtg agt ttc acc      784
Ile Glu Asn Met Phe Phe Val Ser Ala Asn Pro Trp Val Ser Phe Thr
    140                 145                 150

agt ttt gat tta aac gtg gcc aat atg gac aac ttc ttc gcc ccc gtt      832
Ser Phe Asp Leu Asn Val Ala Asn Met Asp Asn Phe Phe Ala Pro Val
155                 160                 165                 170

ttc acc atg ggc aaa tat tat acg caa ggc gac aag gtg ctg atg ccg      880
Phe Thr Met Gly Lys Tyr Tyr Thr Gln Gly Asp Lys Val Leu Met Pro
                175                 180                 185

ctg gcg att cag gtt cat cat gcc gtt tgt gat ggc ttc cat gtc ggc      928
Leu Ala Ile Gln Val His His Ala Val Cys Asp Gly Phe His Val Gly
            190                 195                 200

aga tgc tta atg aat aca aca gta ctg cga tgagtggcag ggcggggcgt        978
Arg Cys Leu Met Asn Thr Thr Val Leu Arg
        205                 210

aaggcgcgcc atttaaatga agttcctatt ccgaagttcc tattctctag aaagtatagg   1038

aacttcgaag cagctccagc ctacaccctt cttcagggct gactgtttgc ataaaaattc   1098

atctgtatgc acaata                                                   1114


<210> SEQ ID NO 82
<211> LENGTH: 212
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polypeptide

<400> SEQUENCE: 82

Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr Val Asp Ile Ser Gln Trp
1               5                   10                  15
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His Arg Lys Glu His Phe Glu Ala Phe Gln Ser Val Ala Gln Cys Thr
            20                  25                  30

Tyr Asn Gln Thr Val Gln Leu Asp Ile Thr Ala Phe Leu Lys Thr Val
        35                  40                  45

Lys Lys Asn Lys His Lys Phe Tyr Pro Ala Phe Ile His Ile Leu Ala
    50                  55                  60

Arg Leu Met Asn Ala His Pro Glu Leu Arg Met Ala Met Lys Asp Gly
65                  70                  75                  80

Glu Leu Val Ile Trp Asp Ser Val His Pro Cys Tyr Thr Val Phe His
                85                  90                  95

Glu Gln Thr Glu Thr Phe Ser Ser Leu Trp Ser Glu Tyr His Asp Asp
            100                 105                 110

Phe Arg Gln Phe Leu His Ile Tyr Ser Gln Asp Val Ala Cys Tyr Gly
        115                 120                 125

Glu Asn Leu Ala Tyr Phe Pro Lys Gly Phe Ile Glu Asn Met Phe Phe
    130                 135                 140

Val Ser Ala Asn Pro Trp Val Ser Phe Thr Ser Phe Asp Leu Asn Val
145                 150                 155                 160

Ala Asn Met Asp Asn Phe Phe Ala Pro Val Phe Thr Met Gly Lys Tyr
                165                 170                 175

Tyr Thr Gln Gly Asp Lys Val Leu Met Pro Leu Ala Ile Gln Val His
            180                 185                 190

His Ala Val Cys Asp Gly Phe His Val Gly Arg Cys Leu Met Asn Thr
        195                 200                 205

Thr Val Leu Arg
    210
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<210> SEQ ID NO 83
<211> LENGTH: 2521
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 83

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tgccagagtc gtcagcaatg tcatggctcg ttcgcgtaaa gcttgcagtt gatgttggtc    180

tgccgttgca tcacttttcg ccggttgttg tattaatgtt gctaattgat agcaatagac    240

catcaccgcc tgccccagat tgagcgaagg ataatccgcc accatcggca caccagtaag    300

aacgtcagcc aacgctaact cttcgttagt caacccggaa tcttcgcgac caaacaccag    360

cgcggcatgg ctcatccatg aagatttttc ctctaacagc ggcaccagtt caactggcgt    420

ggcgtagtaa tgatatttcg cccgactgcg cgcagtggtg gcgacagtga aatcgacatc    480

gtgtaacgat tcagccaatg tcgggaaaac tttaatatta tcaataatat caccagatcc    540

atgtgcgacc cagcgggtgg ctggctccag gtgtgcctga ctatcgacaa tccgcagatc    600

gctaaacccc atcgttttca ttgcccgcgc cgctgcccca atattttctg ctctggcggg    660

tgcgaccaga ataatcgtta tacgcatatt gccactcttc ttgatcaaat aaccgcgaac    720

cgggtgatca ctgtcaactt attacgcggt gcgaatttac aaattcttaa cgtaagtcgc    780

agaaaaagcc ctttacttag cttaaaaaag gctaaactat ttcctgactg tactaacggt    840
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tgagttgtta aaaaatgcta catatccttc tgtttactta ggataatttt ataaaaaata 900

aatctcgaca attggattca ccacgtttat tagttgtatg atgcaactag ttggattatt 960

aaaataatgt gacgaaagct agcatttaga tacgatgatt tcatcaaact gttaacgtgc 1020

tacaattgaa cttgatatat gtcaacgaag cgtagtttta ttgggtgtcc ggcccctctt 1080

agcctgttat gttgctgtta aaatggttag gatgacagcc gtttttgaca ctgtcgggtc 1140

ctgagggaaa gtacccacga ccaagctaat gatgttgttg acgttgatgg aaagtgcatc 1200

aagaacgcaa ttacgtactt tagtcatgtt acgccgatca tgttaatttg cagcatgcat 1260

caggcaggtc agggactttt gtacttcctg tttcgattta gttggcaatt taggtagcaa 1320

acgaattcat cggctttacc accgtcaaaa aaaacggcgc tttttagcgc cgtttttatt 1380

tttcaacctt atttccagat acgtaactca tcgtccgttg taacttcttt actggctttc 1440

attttcggca gtgaaaacgc ataccagtcg atattacggg tcacaaacat catgccggcc 1500

agcgccacca ccagcacact ggttcccaac aacagcgcgc tatcggcaga gttgagcagt 1560

ccccacatca caccatccag caacaacagc gcgagggtaa acaacatgct gttgcaccaa 1620

cctttcaata ccgcttgcaa ataaataccg ttcattatcg ccccaatcag actggcgatt 1680

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accaatgaaa gccccaccag caaatattgc attgggtgta aacgttgcgc ggtgagcgtt 1800

tcaaaaacaa agaacgccat aaaagtcagt gcaatcagca gaatggcgta cttagtcgcc 1860

cggtcagtta attggtattg atcggctggc gtcgttactg cgacgctaaa cgccgggaag 1920

ttttcccagc cggtatcatt gcctgaagca aaacgctcac cgagattatt agcaaaccag 1980

ctgctttgcc agtgcgcctg aaaacctgac tcgctaactt cccgtttggc tggtagaaaa 2040

tcacctaaaa aactgggatg cggccagttg ctggttaagg tcatttcgct attacgcccg 2100

ccaggcacca cagaaagatc gccggtaccg cttaaattca gggccatatt cagcttcagg 2160

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aacccggtgc cgggttcaat ggtcagcgcc gttccgttaa cttcaggcgc tttcaccaca 2280

ccaataccac gcgcatcccc gacgctaatc acaataaatg gcttgcctaa ggtgatattt 2340

ggcgcgttga gttcgctaag acgcgaaaca tcgaaatcgg cttttaacgt taaatcactg 2400

tgccagacct gaccggtata aatccctatc ttgcgttctt ccacgttctg attgccatca 2460

accatcaatg actcaggtaa ccaaaaatgg ataaaacttc gtttccgctg cagggtttta 2520

t 2521

<210> SEQ ID NO 84
<211> LENGTH: 3010
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 84

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ggacgctatc gccgtgatgg ggaaccggat ggtctgtagg tccagattaa caggtctttg 120

ttttttcaca tttcttatca tgaataacgc ccacatgctg ttcttattat tccctgggga 180

ctacgggcac agaggttaac tttctgttac ctggagacgt cgggatttcc ttcctccggt 240

ctgcttgcgg gtcagacagc gtcctttcta taactgcgcg tcatgcaaaa cactgcttcc 300

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agatgcgaaa acgacacgtt acaacgctgg gtggctcggg attgcagggt gttccggaga 360

cctggcggca gtataggctg ttcacaaaat cattacaatt aacctacata tagtttgtcg 420

ggttttatcc tgaacagtga tccaggtcac gataacaaca tttatttaat ttttaatcat 480

ctaatttgac aatcattcaa caaagttgtt acaaacatta ccaggaaaag catataatgc 540

gtaaaagtta tgaagtcggt atttcaccta agattaactt atgtaacagt gtggaagtat 600

tgaccaattc attcgggaca gttattagtg gtagacaagt ttaataattc ggattgctaa 660

gtacttgatt cgccatttat tcgtcatcaa tggatccttt acctgcaagc gcccagagct 720

ctgtacccag gttttcccct ctttcacaga gcggcgagcc aaataaaaaa cgggtaaagc 780

caggttgatg tgcgaaggca aatttaagtt ccggcagtct tacgcaataa ggcgctaagg 840

agaccttaaa tggctgatac aaaagcaaaa ctcaccctca acggggatac agctgttgaa 900

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gcgaccgatt ctaactacct ggaagtttgt tacatcctgc tgaatggtga aaaaccgact 1140

caggaacagt atgacgaatt taaaactacg gtgacccgtc ataccatgat ccacgagcag 1200

attacccgtc tgttccatgc tttccgtcgc gactcgcatc caatggcagt catgtgtggt 1260

attaccggcg cgctggcggc gttctatcac gactcgctgg atgttaacaa tcctcgtcac 1320

cgtgaaattg ccgcgttcct cctgctgtcg aaaatgccga ccatggccgc gatgtgttac 1380

aagtattcca ttggtcagcc atttgtttac ccgcgcaacg atctctccta cgccggtaac 1440

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cgtgctatgg accgtattct gatcctgcac gctgaccatg aacagaacgc ctctacctcc 1560

accgtgcgta ccgctggctc ttcgggtgcg aacccgtttg cctgtatcgc agcaggtatt 1620

gcttcactgt ggggacctgc gcacggcggt gctaacgaag cggcgctgaa aatgctggaa 1680

gaaatcagct ccgttaaaca cattccggaa tttgttcgtc gtgcgaaaga caaaaatgat 1740

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tccatgttca ccgtcatttt cgcaatggca cgtaccgttg gctggatcgc ccactggagc 2040

gaaatgcaca gtgacggtat gaagattgcc cgtccgcgtc agctgtatac aggatatgaa 2100

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agcaacccaa tactttcaat taaaaacttc atggtagtcg catttataac cctatgaaaa 2340

tgacgtctat ctatacccca ctatatttta ttcatcatac aacaaattca tgataccaat 2400

aatttagttt tgcatttaat aaaactaaca atatttttaa gcaaaactaa aaactagcaa 2460

taatcaaata cgatattctg gcgtagctat acccctattc tatatcctta aaggactctg 2520

ttatgtttaa aggacaaaaa acattggccg cactggccgt atctctgctg ttcactgcac 2580

ctgtttatgc tgctgatgaa ggttctggcg aaattcactt taaggggggag gttattgaag 2640

caccttgtga aattcatcca gaagatattg ataaaaacat agatcttgga caagtcacga 2700

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caacccatat aaaccgggag catcatagca ataaagtggc cgtcgacatt cgcttgatca 2760

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tcgcctggat 3010

<210> SEQ ID NO 85
<211> LENGTH: 4180
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 85

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gccatcattg gcgaaccgac gtcactacaa ccggtacgcg cacataaagg tcatatctct 180

aacgccatcc gtattcaggg ccagtcgggg cactccagcg atccagcacg cggagttaac 240

gctatcgaac taatgcacga cgccatcggg catattttgc aattgcgcga taacctgaaa 300

gaacgttatc actacgaagc gtttaccgtg ccataccta cgctcaacct cgggcatatt 360

cacggtggcg acgcttctaa ccgtatttgc gcttgctgtg agttgcatat ggatattcgt 420

ccgctgcctg gcatgacact caatgaactt aatggtttgc tcaacgatgc attggctccg 480

gtgagcgaac gctggccggg tcgtctgacg gtcgacgagc tgcatccgcc gatccctggc 540

tatgaatgcc caccgaatca tcaactggtt gaagtggttg agaaattgct cggagcaaaa 600

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caataatgtc ggatgcgatg cttgcgcatc ttatccgacc tacagtgact caaacgatgc 900

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gttagatgaa aaaaccaaag atactgtttg gtggacatct gaaacagcaa aaaacgacaa 1620

caagccaatg aaccaagcta catggcaaag cttaaaagac ttggtaacca accagctttc 1680

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tcgtaaacgc ttatttgtag ttgatggttt ctgtggtgcg agcgaacacg accgtattgc 1740

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agtaaccaat ccaaactgga aagaacaagg tttaaattca gaaaactttg ttgctttcaa 1920

cttgactgaa cgcattcaat taatcggtgg tacttggtac ggcggtgaaa tgaaaaaagg 1980

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agaaaatgaa ccagatattt accgcgctat ccgtcgcgac gcattattag aaaacgtggt 2280

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<210> SEQ ID NO 86
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 86

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<210> SEQ ID NO 87
<211> LENGTH: 5083
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 87

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<210> SEQ ID NO 88
<211> LENGTH: 5097
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 88

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What is claimed is:

1. A non-naturally occurring *Escherichia coli*, comprising a 1,4-butanediol (BDO) pathway comprising one or more heterologous polynucleotides encoding BDO pathway enzymes expressed in a sufficient amount to produce BDO, wherein said *E. coli:*

(A) comprises a BDO pathway comprising:

(a) alpha-ketoglutarate decarboxylase; or alpha-ketoglutarate dehydrogenase and CoA-dependent succinate semialdehyde dehydrogenase; or glutamate:succinate semialdehyde transaminase and glutamate decarboxylase;

(b) 4-hydroxybutyrate dehydrogenase;

(c) 4-hydroxybutyryl-CoA transferase; or 4-hydroxybutyrate kinase and phosphotrans-4-hydroxybutyrylase; and

(d) 4-hydroxybutyryl-CoA reductase and 4-hydroxybutyraldehyde reductase; or aldehyde/alcohol dehydrogenase, said aldehyde/alcohol dehydrogenase converting 4-hydroxybutyryl-CoA to 1,4-butanediol; and

(B) comprises disruption of a gene encoding a protein in an aerobic respiratory control regulatory system; or expresses an exogenous NADH insensitive citrate synthase.

2. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* comprises disruption of a gene encoding a protein in an aerobic respiratory control regulatory system.

3. The non-naturally occurring *E. coli* of claim **2**, wherein said gene encoding the protein in the aerobic respiratory control regulatory system is an arcA gene.

4. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* expresses an exogenous NADH insensitive citrate synthase.

5. The non-naturally occurring *E. coli* of claim **4**, wherein said NADH insensitive citrate synthase is encoded by a gltA gene or a mutant gltA gene encoding an R163L mutant NADH insensitive citrate synthase.

6. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* further expresses an exogenous phosphoenolpyruvate carboxykinase.

7. The non-naturally occurring *E. coli* of claim **1**, further comprising disruption of a gene encoding malate dehydrogenase.

8. The non-naturally occurring *E. coli* of claim **1**, wherein one or more of said one or more heterologous nucleotides encoding BDO pathway enzymes are integrated into the fimD locus of the *E. coli.*

9. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* further expresses an exogenous non-phosphotransferase sucrose uptake system.

10. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* further comprises disruption of endogenous lactate dehydrogenase, endogenous alcohol dehydrogenase, and endogenous pyruvate formate lyase.

11. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* further expresses an exogenous pyruvate dehydrogenase.

12. The non-naturally occurring *E. coli* of claim **11**, wherein one or more genes encoding pyruvate dehydrogenase subunits is under the control of a pyruvate formate lyase promoter.

13. The non-naturally occurring *E. coli* of claim **11**, wherein said exogenous pyruvate dehydrogenase is NADH insensitive.

14. The non-naturally occurring *E. coli* of claim **11**, wherein said exogenous pyruvate dehydrogenase is encoded by the *Klebsiella pneumonia* lpdA gene.

15. The non-naturally occurring *E. coli* of claim **1**, wherein said succinate semialdehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA/acetyl-CoA transferase are encoded by *Porphyromonas gingivalis* W83 genes.

16. The non-naturally occurring *E. coli* of claim **1**, wherein said 4-hydroxybutyryl-CoA reductase is encoded by the *Clostridium beijerinckii* ald gene.

17. The non-naturally occurring *E. coli* of claim **1**, wherein said *E. coli* further expresses an exogenous succinyl-CoA synthetase.

18. The non-naturally occurring *E. coli* of claim **17**, wherein said succinyl-CoA synthetase is encoded by the *Escherichia coli* sucCD genes.

19. The non-naturally occurring *E. coli* of claim **1**, wherein said alpha-ketoglutarate decarboxylase is encoded by the *Mycobacterium bovis* sucA gene.

20. The non-naturally occurring *E. coli* of claim **1**, wherein said 4-hydroxybutyrate kinase and said phosphotrans-4-hydroxybutyrylase are encoded by the *Clostridium acetobutylicum* buk1 and ptb genes.

21. The non-naturally occurring *E. coli* of claim **1**, wherein said 4-hydroxybutyryl-CoA reductase is encoded by the *Clostridium beijerinckii* ald gene.

22. The non-naturally occurring *E. coli* of claim **1**, wherein said 4-hydroxybutyraldehyde reductase is encoded by the *Geobacillus thermoglucosidasius* adh1 gene.

23. The non-naturally occurring *E. coli* of claim **6**, wherein said phosphoenolpyruvate carboxykinase is encoded by the *Haemophilus influenza* phosphoenolpyruvate carboxykinase gene.

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